THE HYPERGLYCEMIC HORMONE PRODUCING SYSTEM IN THE EYESTALK OF THE CRAYFISH *ASTACUS LEPTODACTYLUS*

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In order to coordinate the activities of cells, tissues and organs and to adapt biological processes adequately to changing environmental conditions, most metazoan species are equipped with a nervous system which is particularly well adapted for rapid communication and an endocrine system which is more suited to control relatively long-term bodily processes. Nerve cells function, except where they make electrical synapses, by exhibiting electrical activity which results in the release of a neuromodulator from the axon terminal in the close neighbourhood of the target cells. These neuromodulators or neurotransmitters - mostly substances of low molecular weights (e.g. acetylcholine, noradrenaline, adrenaline, dopamine)- are produced in little amounts and most probably are reabsorbed for reusal. Endocrine cells release their synthesized products - hormones, representing peptides or (glyco)proteines with relatively high molecular weights, or steroids - into the circulatory system by which they will be transported to the more distantly located target cells. As a consequence, hormones have to be synthesized and released in relatively much larger amounts than neuromodulators.
These two coordinating systems are bridged by a third one, the neurosecretory system. This consists of specialized nerve cells, called neurosecretory cells, which primary function is the synthesis and release of so-called neurohormones. Neurosecretory cells are easily recognized at the light and electron microscopic levels by their pronounced glandular features and by the fact that, instead of establishing synaptic contact with contiguous effector cells, they terminate in close proximity to the vascular system, into which they release their products. Groups of nerve terminals filled with neurosecretory storage material, often form a socalled neurohemal organ with the vascular system. The secretory products usually are of a proteinacous nature and, once they have been released into the blood, act like hormones.

In recent years, important contributions to this core of knowledge have yielded new insights as a result of which the dividing line between conventional and neurosecretory neurons is no longer sharp as it appeared in the past. Recent studies revealed the neuromodulating function of peptides (e.g. Emson 1979; Adams and O'Shea 1983) while neurosecretory cells have been described producing substances that chemically resemble those operating as neurotransmitters (Scharrer and Weitzman 1970). Indications were further obtained for the existence of peptidergic neurons that also produce bioamines (e.g. Osborne and Dockray 1982). Moreover, terminals of neurosecretory cells have been described that are so close to endocrine cells that the dispatch of neurosecretory signals does not involve the circulatory system (e.g. B. Scharrer 1964a,b; Wendelaar Bonga 1971).
Furthermore, Pearse (1966, 1976) put new light on the ontogenetic relationship between peptidergic epithelial gland cells and neurosecretory cells, by introducing his APUD concept. This acronym stands for Amine Precursor Uptake and Decarboxylation, which describes their cytochemical properties.
For reviews of the above presented fields of interest see Bennett (1977), Berlind (1977), Gainer (1977), B. Scharrer (1970, 1978, 1983) Maddrell and Nordmann (1979), Hökfelt et al. (1980), Goldsworthy et al. (1981), Karlson (1982).

The study on neurosecretory cells goes back to the second half of the nineteenth and the beginning of the twentieth century. By that time neurosecretory cells were described, however, without recognizing their glandular features. Most of this work relates to invertebrates. In fact Bellonci (1882a,b) was the first who figured neurosecretory cells in the X-organs of some crustaceans (for a review of these pioneer studies see Gabe 1966).

The phenomenon of neurosecretion was formulated for the first time in the late 1920's and early 1930's when E. Scharrer definitely established the existence of nerve cells in the hypothalamic-hypophyseal system of vertebrates. In addition to their neuronal characteristics, these cells exhibited the main attribute of a gland cell: the elaboration of a secretory product. The extension of this type of observations to invertebrates was very rapid. In 1931 Hanström described the presence of neurosecretory cells in the eyestalk of several decapod crustaceans, which was soon followed by the description of neurosecretory cells in molluos, annelids and an insect. For reviews of the first studies on neurosecretion is referred to E. Scharrer and B. Scharrer (1937, 1945, 1954). Since these pioneer studies, investigations on neurosecretion assumed large proportion. By now, neurosecretory neurons are found widely in the animal kingdom, from primitive metazoans to mammals (for reviews see Gabe 1966; Berlind 1977; Morris et al. 1978).

The most widely studied among these organ complexes is the hypothalamic-neurohypophyseal system of vertebrates. Most neurohormones released by this system have a modulating function concerning the regulation of endocrine glands located elsewhere in the body. In invertebrate endocrinology, neurosecretory cells occupy a more dominant position. As these animals do possess no or only few peripheral endocrine glands, neurosecretory cells are involved directly in the regulation of most biological processes. In lower invertebrates neurosecretory cells are distributed throughout the body or the nervous system, but in higher invertebrates, such as molluscs and arthropods, there is a tendency for neurosecretory cells to be concentrated in the cerebral ganglia.

The neurosecretory system as present in crustaceans (Malacostraca) consists of neurosecretory cells mainly located in the protocerebrum and the cerebral ganglia. Furthermore, some cells are found in the sub-oesophageal ganglion and throughout thoracic and abdominal ganglia. The main neurohemal organs are the sinus gland, the postcommissural organs and the pericardial organs.

The most important neurosecretory center is the protocephalic X-organ-sinus gland complex, which is responsible for the production of the majority of the known regulating neurohormones. Hanström (1931, 1933) was the first who reported the presence of neurosecretory cells in the eyestalk of several species of decapod crustaceans. The same papers deal with the description of the sinus gland, which by that time was considered as a conventional gland responsible for production, storage and release of secretory products. Hanström in his first reports already described a nervous connection between the X-organ and the sinus gland. The true relationship between both was not revealed until twenty years later in 1951 when Bliss, Passano and Enami independently presented evidence establishing the sinus gland as a neurohemal region, where the secretory products of the X-organ cell bodies were released into the hemolymph. Studies in a number of laboratories in the next few years established that all the known hormonal substances present in the sinus gland are also present in the X-organ; for a review and a summary of the historical background of neurosecretion see Gabe (1966).

Most morphological and physiological investigations on the X-organ sinus gland complex have been carried out on decapod crustaceans. Hanström (1928) divided the eyestalks of decapod crustaceans in four types on the basis of the location of the protocephalic ganglia and the optic center. The eyestalks of the crayfish *Astacus leptodactylus*, the species used as main experimental animal for the research presented in this thesis belong to the so-called "*Carcinus*" type. This implies that the optic ganglia (lamina ganglionaris, medulla externa, medulla interna and medulla terminalis) are located within the eyestalk. They are connected with the cerebral ganglion by means of the optic nerve. The majority of neurosecretory cells in this species is found in the medulla terminalis where they are concentrated mainly on the

rostral latero-ventral side. They are indicated as Medulla Terminalis Ganglionic X-organ (MTGX). The axons of these cells form a tract which runs to the sinus gland located dorso-laterally at the transition of the medulla interna and medulla externa. A diagram of the location of the optic ganglia and the X-organ sinus gland system in the eyestalk of decapod species belonging to the ***Carcinus***- type is presented in Fig. 1, Chapter II. For a review on the organization of the crustacean optic lobes and the morphology of the X-organ sinus gland system see Sandeman (1982) and Cooke and Sullivan (1982).

The X-organ sinus gland system represents the most important source of neurosecretory products which are involved in the regulation of a variety of biological processes such as moulting, reproduction, pigment migration, osmoregulation and carbohydrate metabolism (for reviews see Kleinholz and Keller 1979; Cooke and Sullivan 1982).

One of these neurosecretory products is the Crustacean Hyperglycemic Hormone (CHH), involved in the regulation of the hemolymph glucose concentration and the glycogen metabolism. Abramowitz and his coworkers (1944) were the first who described an increase in the blood sugar level after injection of eyestalk extract. They assumed the presence of a "diabetogenic factor" in the sinus gland. In the following years their findings were confirmed by other investigators for various decapod species (Kleinholz and Little 1949; Kleinholz et al. 1950; Scheer and Scheer 1951). The term "hyperglycemic hormone" was introduced in 1967 by Kleinholz and coworkers. From that time more information became available concerning the biochemical nature and interspecific physiological differences of the CHH and the regulation of its synthesis and release at the organ and cellular level.

Initial studies characterized the hyperglycemic hormone as a polypeptide with a molecular weight around 6000-7000 D (Kleinholz 1966; Kleinholz et al. 1967; Keller 1968; Kleinholz and Keller 1973). By now the amino-acid composition of CHH has been determined for several decapod species (Kleinholz 1975; Keller and Wunderer 1978; Keller 1981; Newcomb 1983; Keller and Kegel 1984) and an isopod species (Martin et al. 1984). Several authors described more than one molecular form of the CHH, and the existence of a prohormone or precursor has been proposed (Kleinholz and Keller 1973; Keller 1977; Skorkowski et al. 1977; Newcomb 1983; Stuenkel 1983; Van Wormhoudt et al. 1984a,b).

Systematic group specificity for the biological activity and molecular structure of this neurohormone has been reported by Keller (1968; 1969; 1977), Kleinholz and Keller (1973) and Leuven et al. (1982).

Studies on the release of CHH demonstrated the possible role of serotonin and d-c-AMP in the exocytotic release of the CHH granules (Keller and Beyer 1968; Strolenberg and Van Herp 1977; Martin 1968; Strolenberg 1979; Van Herp and Strolenberg 1980).

Concerning the mode of action of the CHH, it has been proposed that this hormone exerts hyperglycemia by depleting glycogen stores in several tissues like abdominal muscles, gonads, integument, gills and hepatopancreas (Schwabe et al. 1952; Keller 1965; Parvathy 1972; Keller and Andrew 1973; Telford 1975). Phosphorylase and glycogen synthetase are two enzymes through which CHH may act on these target organs (Wang and Scheer 1963; Keller 1965, 1966; Bauchau et al. 1968; Sedlmeier 1982). Sedlmeier and Keller (1981) reported a stimulating influence of CHH on the level of cyclic nucleotides in the target organs.

Reviews on the characteristics of CHH, its mode of action and its physiological role were presented by Keller (1974), Kleinholz (1976), Kleinholz and Keller (1979), Chang and O'Connor (1983) and Keller et al. (1983).

Until the late 1970's, information was missing on the location, morphology and physiological functioning of the CHH-producing system. Information on these

topics did not become available until the localization of the CHH-producing cell bodies was established. This became possible after the introduction of immunocytochemical staining techniques. The first data on the location of the CHH-producing cells, the course of the axonal tract and the description of CHH-containing axon terminals in the sinus gland, were presented for the crayfish *Astacus leptodactylus* (Van Herp and Van Buggenum 1979) and for the crab *Carcinus maenas* (Jaros and Keller 1979).

The present study is aimed to collect more information on the location, morphology and innervation of the CHH-producing neurosecretory cells and the appearance of the CHH system under different physiological conditions and biological cycles. Furthermore, a pilot study is presented on the biochemical nature of the hyperglycemic hormone as present in the CHH system e.g. the presence of a prohormone or precursor.

In Chapter I the localization of the CHH-producing perikarya, the CHH-transporting axons and the CHH-containing axon terminals in the sinus gland are described by light, fluorescence and electron microscopy. CHH was vizualised by means of an antiserum raised against a biologically active hyperglycemic fraction of the sinus gland, combined with the unlabeled peroxidase-antiperoxidase staining technique (PAP-method) developed by Sternberger (1974).

In a comparative study on the location of the CHH system in different species, results are obtained that allow us to draw general conclusions on the location of the CHH system in the eyestalk of decapod crustaceans (Chapter II).

Functional aspects of the secretory activity stages of the CHH cells are studied by means of immunocytochemistry combined with morphologic and morphometric investigation. The results indicate a correlation between the intensity of the immunoreactivity of an individual CHH cell and its secretory activity (Chapter III).

To learn more about the functioning of the CHH system during biological cycles, the secretory dynamics of the CHH cells and the hemolymph glucose rhythmicity, are studied during the day/night cycle (Chapter IV) and during the moulting period (Chapter V). In this last above-mentioned chapter, preliminary results are presented on the modulating effect of ecdysteroids on the daily blood glucose rhythm. Furthermore, the CHH-producing cells and the CHH-containing granules in the sinus gland are examined during larval and post-larval development (Chapter VI).

Indications for input on the CHH cells is studied by injection of the fluorescent dye Lucifer Yellow, which reveals the shape of single cells. Electron microscopic investigations demonstrate synaptic input on CHH axon ramifications (Chapter VII).

A pilot study concerning the biochemical nature of the CHH in the X-organ sinus gland system presents indications for multiple forms which may result from functional molecular variants such as a prohormone or precursor (Chapter VIII).

CHAPTER I

IMMUNOCYTOCHEMICAL IDENTIFICATION OF THE CRUSTACEAN HYPERGLYCEMIC HORMONE IN THE X-ORGAN SINUS GLAND COMPLEX OF THE CRAYFISH ASTACUS LEPTODACTYLUS

Gorgels-Kallen and Van Herp (1981),
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Réunion des Carcinologistes de Langue Française,
Banyuls-sur-Mer (01.06-06.06.1981):
Van Herp and Gorgels-Kallen (1981),
and presented at the scientific meeting of the
Nederlandse Zoölogische Vereniging,
Groningen (11.09.1981): Gorgels-Kallen (1982).

SUMMARY

The eyestalk of the crayfish *Astacus leptodactylus* has been investigated immunocytochemically by light, fluorescence and electron microscopy, with an antiserum raised against purified crustacean hyperglycemic hormone (CHH).

CHH can be visualized in a group of neurosecretory perikarya on the medulla terminalis (medulla terminalis ganglionic X-organ. MTGX), in fibers forming part of the MTGX-sinus gland tract, and in a considerable part of the axon terminals composing the sinus gland. Immunocytochemical and ultrastructural investigations led to the identification of the CHH-producing cells and the CHH-containing neurosecretory granule type.

INTRODUCTION

The X-organ sinus gland complex, an important neurosecretory system in the eyestalk of decapod crustaceans belonging to the so-called "*Carcinus*" type as described by Hanstrom (1928), is characterized by neurosecretory cells, the X-organ, of which axon terminals constitute the neurohemal organ, called the sinus gland.

Light microscopic studies and investigations by means of cobalt iontophoresis have demonstrated that most of the axons terminating in the sinus gland originate in neurosecretory perikarya situated on the medulla terminalis (medulla terminalis ganglionic X-organ: MTGX), see e.g. Durand (1956), Gabe (1966), Andrew et al. (1978), Jaros (1978). Electron microscopic studies have demonstrated that the substances synthesized in the perikarya are packed as membrane bound granules and transported through the tract to the sinus gland where they can be stored before being released into the hemolymph The axon terminals comprising the sinus gland, can be divided in several types, on the basis of differences in diameter, shape and electron density of the granules (Fingerman and Aoto 1959, Bunt and Ashby 1967; Phatak and Rangneker 1976; Strolenberg et al. 1977a,b)

Biochemical and physiological investigations revealed that the X-organ sinus gland complex is responsible for the production of at least six different hormonally active substances One of these neurohormones, for the first time described by Abramowitz et al. (1944) as a "diabetogenic factor", is the crustacean hyperglycemic hormone (CHH). For a review see Kleinholz and Keller (1979).

Several studies have been undertaken to learn more about the location of the CHH-producing system in the X-organ sinus gland complex Strolenberg et al. (1977b) described five different types of neurosecretory granules in the sinus gland of the crayfish *Astacus leptodactylus*. From injection experiments, with either serotonin or d-c-AMP, combined with a quantitative electron microscopic study of the sinus gland, the release of CHH could be related to exocytosis of granule type IV and/or V (Strolenberg and Van Herp 1977; Strolenberg 1979; Van Herp and Strolenberg 1980). Van Herp and Van Buggenum (1979) were able to localize by light microscopy the CHH-producing neurosecretory cells in the eyestalk of *Astacus leptodactylus*, applying the PAP staining technique (method described by Sternberger 1974) in combination with a primary antiserum against an *Astacus* hyperglycemic hormone preparation. By both light and electron microscopy, Jaros (1979) demonstrated neurosecretory material in the MTGX-sinus gland complex of the crab *Carcinus maenas*, using an antiserum against sinus gland extract in combination with the PAP technique Jaros and Keller (1979) reported the direct demonstration of CHH in the eyestalk of *Carcinus maenas* with this anti-sinus gland serum in the double antibody fluorescence technique

In the present study a complete immunocytochemical description is given by light, fluorescence and electron microscopy, of the site of production, storage and release of CHH in the eyestalk of *Astacus leptodactylus*.

MATERIALS AND METHODS

Animals

Crayfish of the species *Astacus leptodactylus* (Nordmann 1842), were imported from Turkey via a commercial dealer (Halewijck, Oostende) and kept in the laboratory in running tap water (13-15°C), under constant conditions of 12 h light and 12 h dark. Eyestalks, MTGX-organs, tracts and sinus glands were dissected from normally fed, adult, male crayfish that were in stage C of their moulting cycle (Drach 1944).

Antiserum

The CHH of *Astacus leptodactylus* was isolated from total lyophilized eyestalks by a combination of the extraction and preliminary fractionation steps (described by Kleinholz and Keller 1973), preparative polyacrylamide gel electrophoresis (according to the disc gel electrophoresis of Davis 1964) and gel filtration on Sephadex G-75 (fine and superfine).
To obtain antisera against the biologically active hyperglycemic hormone preparation, New Zealand White albino rabbits were immunized. Each rabbit received a first intracutaneous injection into 20 different sites of the back with an emulsion of the hormone preparation (225 µg protein after Lowry et al. 1951) and complete Freund's adjuvant (1:1). In addition, a subcutaneous injection into the shoulders and the region of the groins was given 14 and 30 days later with 225 µg CHH emulsified in incomplete Freund's adjuvant (1:1). Ten days later, a booster injection of 65 µg CHH in the last emulsion was injected intracutaneously and on the 63rd day blood was collected by heart puncture.
The specificity of the antigen-antibody reaction (antiserum specificity) was controlled by the micromodification of Ouchterlony's double immunodiffusion test; micro-immunoelectrophoresis; selective immunoprecipitation of the CHH protein in a dilution series followed by a ringtest and a bioassay on the supernatant; a combination of disc electrophoresis and double immunodiffusion (as described by Zwisler and Biel 1966).

Immunocytochemistry at the light microscopic level

Eyestalks were fixed in Bouin-Hollande fluid (24 h) containing 10% of a saturated aqueous solution of sublimate. The fixed material was dehydrated, cleared and embedded in paraplast (57°C) according to the conventional histological procedures. Sections (7 µm) were deparaffinized, washed in lugol and a hyposulfite solution, rinsed in distilled water and equilibrated in 0.05 M Tris-HCl buffered saline, pH 7.6.
The immunocytochemical staining technique was based on the method (PAP staining) described by Sternberger (1974, 1979) and performed as described

by Van Herp and Van Buggenum (1979) with little modification: a dilution of 1/150 was used for the primary antiserum in an overnight incubation at 4°C; GAR/IgG (H+L) (goat-anti-rabbit; Nordic Immunology, Tilburg) and PAP (peroxidase-antiperoxidase; UCB) were used in a dilution of 1/50 and 1/300, respectively, at room temperature; 4-Cl-1-Naphtol/peroxide was used as peroxidase substrate. Tris-HCl buffered saline was used for the dilutions of the reagents and the intrajacent washing steps. Embedding was done in Chrome Glycerin Jelly.
For the immunofluorescent staining 7 µm sections were incubated with the primary antiserum (dilution 1/30; incubation overnight at 4°C and sheep-anti-rabbit-FITC (Statens Bakteriologiska Laboratorium; dilution 1/30; incubation 30 min at room temperature or 37°C). Phosphate buffered saline (PBS) was used for the dilutions of the reagents and the intrajacent washing steps. Embedding was done in 10% glycerol; a Zeiss Universal microscope was used for fluorescence microscopy with an Osram HBO 50 W lamp as light source and UG 1 exciter filter and LP 418 barrier filter.

The specificity of the immunochemical staining was tested by substituting the successive incubations by buffer and by substituting the antiserum by normal rabbit serum (NRS).

Immunocytochemistry at the electron microscopic level

For the immunocytochemical investigation, parts of the medulla terminalis including the MTGX, tracts and sinus glands were dissected and fixed in 2% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 8; 0.2 M sucrose) for 3 h at room temperature. After rinsing in buffer (16 h at 4°C) and dehydration in ethanol or aceton, the tissue was embedded in a mixture based on Epon 812 (Luft 1961). The immunocytochemical staining was performed according to the postembedding staining procedure described by Moriarty and Halmi (1972) and Sternberger (1974), with the primary antiserum in a dilution 1/1000. Incubation with the antiserum took place 48 h at 4°C. Some corresponding grids were counterstained with 2% aqueous uranyl acetate (24 h at 4°C).

The specificity of the immunocytochemical staining was tested by substituting the successive incubations by buffer and by substituting the antiserum by NRS.

Sections were examined in a Philips 300 electron microscope at 60 kV or 40 kV (after PAP staining). For the quantitative work the electron microscope was calibrated with a carbon replica grating grid (2160 lines per mm).
The diameters of the granules which reacted possitively with the primary antiserum were measured from photographic prints (magnification ±30.000×) by use of Hardware Kontron MOB equipment (Kontron Messgeräte GmbH).
The data were submitted to the Student-t-test (two sided; significance accepted when $P<0.05$).

RESULTS

The immunocytochemical study of the eyestalk of the crayfish ***Astacus leptodactylus***, at the light microscopic level, by the PAP staining method and the antibody fluorescence technique, reveals a positive reaction for a number of neurosecretory cells in the MTGX, in the MTGX-sinus gland tract and in a considerable part of the sinus gland (Figs 1-5). Most of the CHH-containing cells form a distinct group, consisting of about 35 cells (diameter 35-50 µm)

situated at the most distal part at the rostral latero-ventral side of the MTGX, while a few others are spread throughout the proximal part (Fig 3). The CHH is visualized as granular aggregated material, distributed at random throughout the cytoplasm. In the X-organ sinus gland tract a group of fibers shows a positive immunoreaction (Fig 2). This immunopositive tract can be followed starting from the CHH-producing perikarya, passing through the rostro-lateral side of the medulla terminalis and running to the region of the sinus gland along the latero-dorsal side of the medulla interna. In the animals investigated, one part of the positively reacting cells shows a great number of strongly stained aggregations, while the other part demonstrates a less dense immunoreaction (Fig 4). Contrastingly, the immunoreaction in the sinus gland is always very strong and homogeneously spread over the neurohemal region as aggregated droplets (Fig 5).

The electron microscopic study confirms the above-mentioned results. After PAP staining CHH-containing granules can be observed in a number of perikarya of the MTGX, in the tract and in the sinus gland. The cytoplasm of the immunopositive cells is characterized by a number of Golgi complexes surrounded by a group of CHH-containing granules (Figs 6-8). The content of the sacculus on the concave side of the Golgi complex often shows an immunopositive reaction (Fig 6, arrows). Part of the positively reacting perikarya shows a great number of Golgi complexes surrounded by many immunoreactive granules (Fig 6), while other cell bodies contain Golgi zones surrounded by only a few granules. The quantitative data on these granules in the MTGX are summarized in Table 1. In those perikarya the mean diameter of the immunopositive granules is 130 nm.

As shown in figures 9 and 10, a positive immunoreaction is also obtained in the MTGX-sinus gland tract. After measurement of the immunoreactive granules in the tract, we find a mean diameter of around 139 nm (Table 1).

As demonstrated by figures 13 and 14, the immunopositive reaction in the sinus gland allows characterization of distinct axon terminals. Examination of the sinus gland reveals that the immunoreaction is correlated to a granule type with a diameter of about 143 nm (Table 1). The increase in mean diameter of the granules from the perikarya to the sinus gland is significant.

The specificity tests of the immunocytochemical staining reveal no aspecific staining both for the light microscopic as well as for the electron microscopic observations (Figs 11,12).

TABLE 1

Morphometric data of the CHH-containing granules in the MTGX, the tract and the sinus gland of the crayfish *Astacus leptodactylus*.

	number of measured granules	mean diameter (nm)	S.E.M.
perikarya (n=3)	160	130	2.1
tract (n=4)	245	139	1.7
sinus gland (n=5)	4500	143	0.3

n = number of animals investigated (per animal one eyestalk); the mean diameter of the granules in the perikarya and the sinus gland differ significantly (Student-t-test, two sided; $P<0.001$).

TABLE 2

Comparison of the granule types occurring in the sinus gland of *Astacus leptodactylus*, measured by Strolenberg et al. (1977) and indicated as *, with data of the CHH-containing granules measured after PAP-staining.

type	diameter (min - max values: nm)	mean diameter (nm)
*I	50 - 75	68
*II	65 - 95	81
*III	85 - 115	100
*IV	100 - 170	112
*V	125 - 220	135
"CHH"	115 - 240	143

DISCUSSION

The results of the present immunocytochemical investigation on the sites of production, storage and release of CHH in the eyestalk of *Astacus leptodactylus*, by light, fluorescence and electron microscopy, confirm the preliminary results of the light microscopic immunocytochemical study of Van Herp and Van Buggenum (1979) on this species On the basis of location, size, outline and staining with the aldehyde fuchsin-trichrome staining procedure, two neurosecretory cell types can be distinguished in the MTGX of *Astacus leptodactylus*. This study demonstrates that the cells described as the neurosecretory cell type 1, represent the hyperglycemic hormone producing cells

Light microscopically, by means of the peroxidase-antiperoxidase (PAP) staining method as well as the double antibody fluorescence staining technique, the CHH can be visualized as granular aggregated material, spread at random in the cytoplasm and as granular "clumps" of immunopositive material in the sinus gland Comparing these light microscopic pictures with the ultrastructural data, we can conclude that the aggregations in the perikarya and the sinus gland correspond to the Golgi complexes in the cytoplasm surrounded by immunopositive granules and the axon terminals in the sinus gland filled up with immunopositive granules, respectively

The CHH cells show differences in immunoreaction This appears light microscopically from the difference in staining intensity and electron microscopically from the observation of cells containing varying numbers of neurosecretory granules in their cytoplasm We suppose that these differences in immunoreactivity are related to different stages in the secretory process of the CHH cells and so to different amounts of antigen in the cytoplasm This phenomenon has been studied and discussed in more detail in Chapter III

Strolenberg et al (1977) described five types of neurosecretory axon terminals in the sinus gland of *Astacus leptodactylus*, on the basis of diameter, electron density and shape of the granules Injection experiments with either serotonin or d-c-AMP revealed an increase in the number of exocytoses of the granule types IV and V, followed by hyperglycemia Furthermore, in a diurnal cycle with the light period from 9 a m to 9 p m , the higher exocytosis activity of type IV and V observed between 9-10 p m was followed by a rise in the blood glucose level between 11-12 p m These results led to the assumption that the granule types IV and/or V contain the CHH (Strolenberg and Van Herp 1977, Strolenberg 1979, Van Herp and Strolenberg 1980) The mean diameter of type IV and V described by Strolenberg et al (1977) is 112 and 135 nm respectively As presented in this paper, the mean diameter of the CHH-containing granules in the sinus gland is 143 nm, which corresponds to granule type V The mean diameter of the CHH-containing granules is somewhat higher than the mean diameter of granule type V Comparison of measurements on sections after PAP staining or counterstaining with uranyl acetate, shows that this difference in mean diameter might derive from the "fuzzy" appearance of the granules after PAP-staining, which does not permite very accurate measurements Furthermore, we need to bear in mind the overlap in the range of the measured diameters of the granule types IV and V, compared with the CHH-containing granules This variance in diameter may be due to individual differences, irregular rates of synthesis or other undefined effects Therefore, in this respect, we conclude that functionally different cell types cannot be distinguished merely on the basis of differences in diameter of their granules However, the present paper illustrates how immunocytochemistry enables a strict identification, so that we can assert the largest granule type to contain the CHH

Keller (1977) and Jaros and Keller (1979) estimated the content of CHH in the sinus gland of *Carcinus maenas* at approximately 10 to 20% of total sinus gland protein. In this respect it is interesting to notice that 20% of the axon terminals composing the sinus gland of *Astacus leptodactylus* are determined as type V (Strolenberg et al. 1977; Strolenberg 1979).

A comparison of the mean diameters of the granules in the perikarya, the tract and the sinus gland indicates a significant growth of the granules during their passage through the tract. A comparable situation was described earlier e.g. by Wendelaar Bonga (1970) for neurosecretory cells in the pond snail *Lymnaea stagnalis*. This increase in granule diameter has been studied in more detail; the results are presented and discussed in the Chapters III and VIII.

ABBREVIATIONS

BM	basement membrane
GC	glial cell
H	hemolymph
LG	lamina ganglionaris
ME	medulla externa
MI	medulla interna
MT	medulla terminalis
MTGX	medulla terminalis ganglionic X-organ
N	nucleus
O	ommatidia
SG	sinus gland
T	tract

LEGENDS

Fig. 1: Longitudinal section of the eyestalk of *Astacus leptodactylus*, showing CHH immunopositive (PAP) reaction in cells of the MTGX and in the sinus gland. Poststaining of the nuclei with hemalum Mayer. Bar represent 100 µm.

Fig. 2: Immunocytochemical (PAP) staining for CHH in the tract, descending from the MTGX, leading to the sinus gland. Bar represents 100 µm.

Fig. 3: Immunocytochemical (PAP) staining of CHH-producing cells in the MTGX. Bar represents 100 µm.

Fig. 4: Immunofluorescent staining for CHH in cells of the MTGX, showing the difference in intensity between individual cells. Bar represents 100 µm.

Fig. 5: Immunofluorescence of CHH material in the sinus gland. Bar represents 100 µm.

Fig. 6: Immunocytochemical (PAP) staining of CHH-producing cells. Notice Golgi complexes (arrows) surrounded by numerous granules. Bar represents 1 µm.

Fig. 7: Immunocytochemical (PAP) staining of two groups of CHH-containing granules. Compare with serial section (Fig 8). Bar represents 1µm.

Fig. 8: Section counterstained with uranyl acetate, showing two Golgi zones, surrounded by CHH-containing granules. Compare with serial section (Fig 7). Bar represents 1 µm.

Fig. 9: Immunocytochemical (PAP) staining for CHH in a section of the MTGX-sinus gland tract. Bar represents 1 µm.

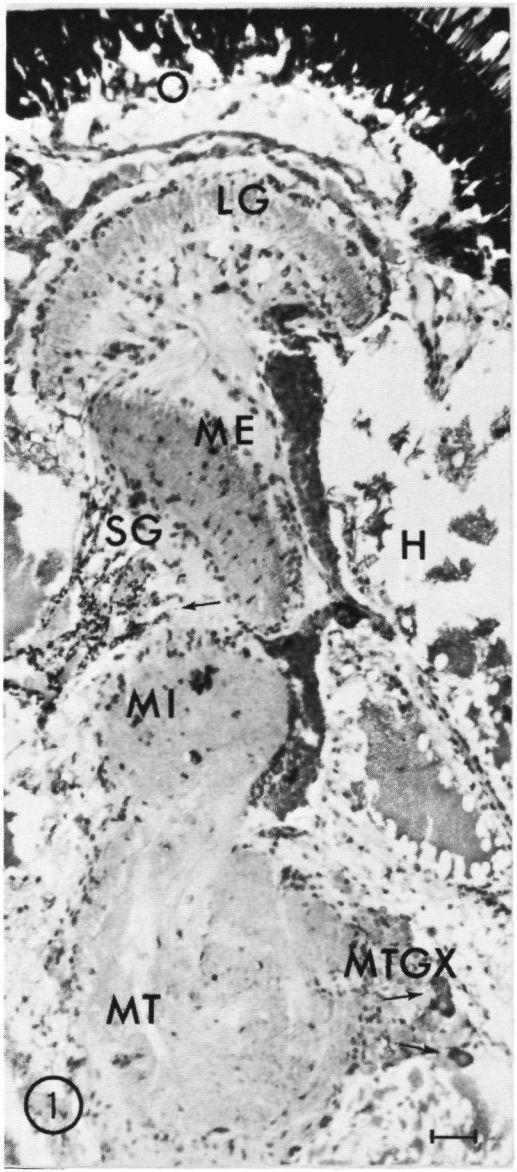
Fig. 10: Section of the MTGX-sinus gland tract counterstained with uranyl acetate. Bar represents 1 µm.

Fig. 11: CHH-containing granules after PAP staining. Bar represents 100 nm.

Fig. 12: CHH-containing granules after testing specificity by substituting the primary antiserum by normal rabbit serum. Bar represents 100 nm.

Fig. 13: Immunocytochemical (PAP) staining in a section of the sinus gland, showing CHH-containing granules in axon terminals. Bar represents 1 µm.

Fig. 14: View of the sinus gland after counterstaining with uranyl acetate. * indicates the granule type V. Bar represents 1 µm.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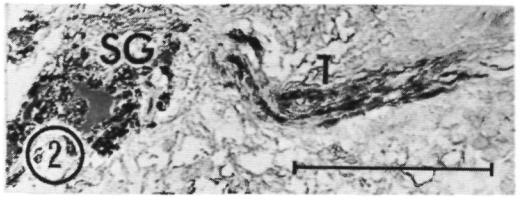
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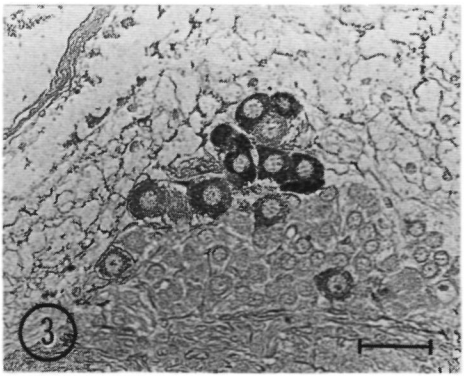
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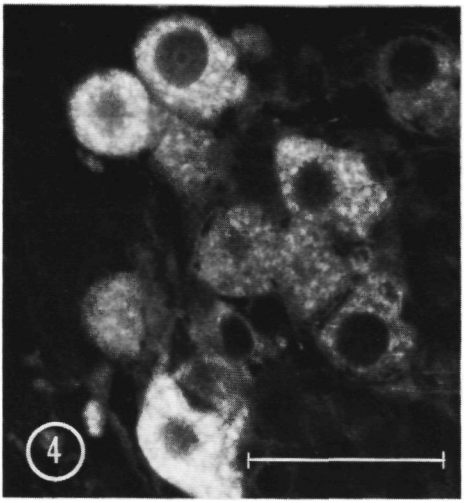
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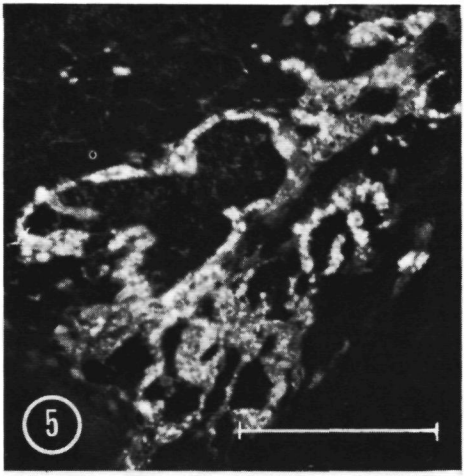
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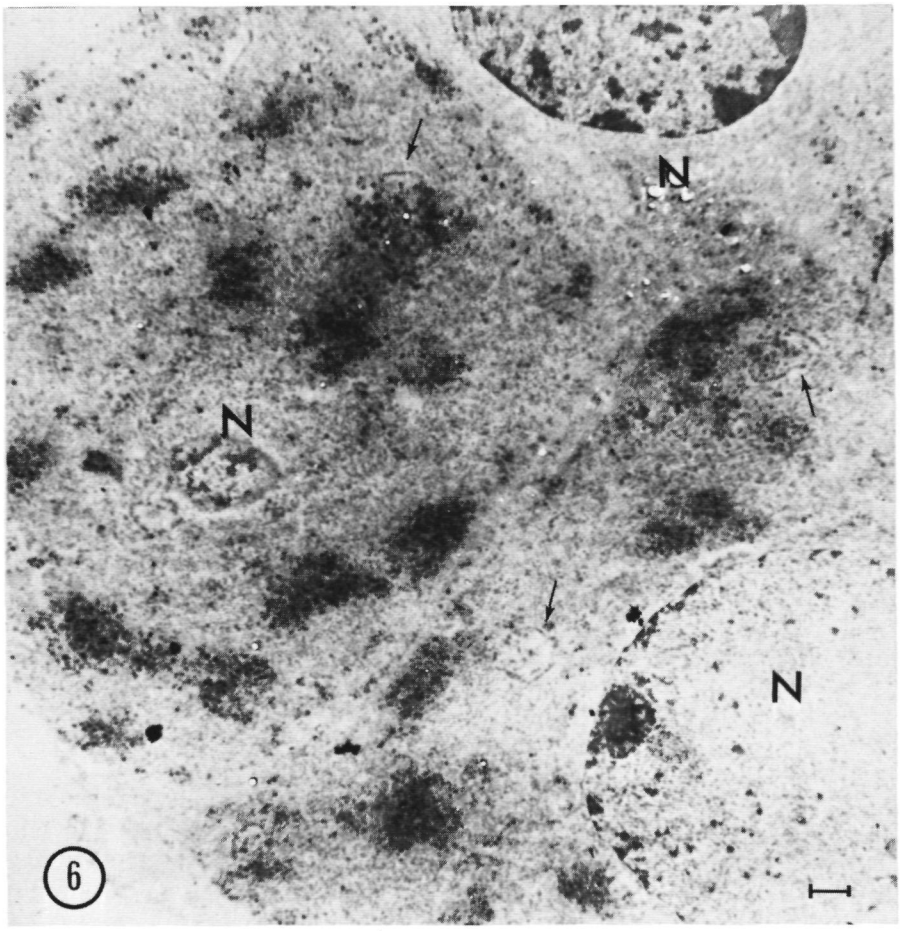
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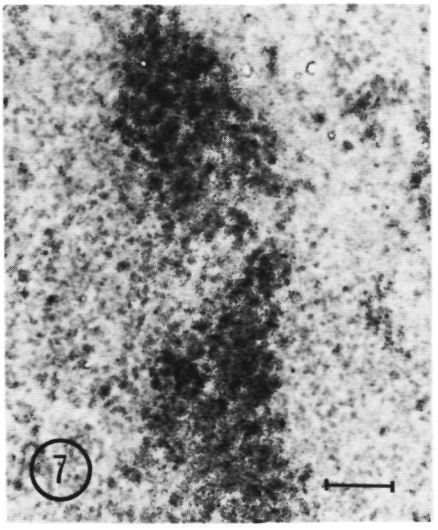
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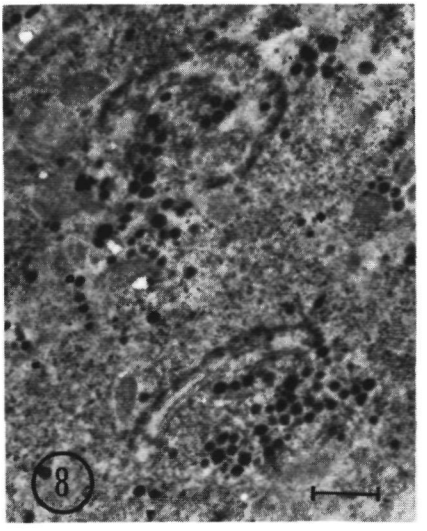
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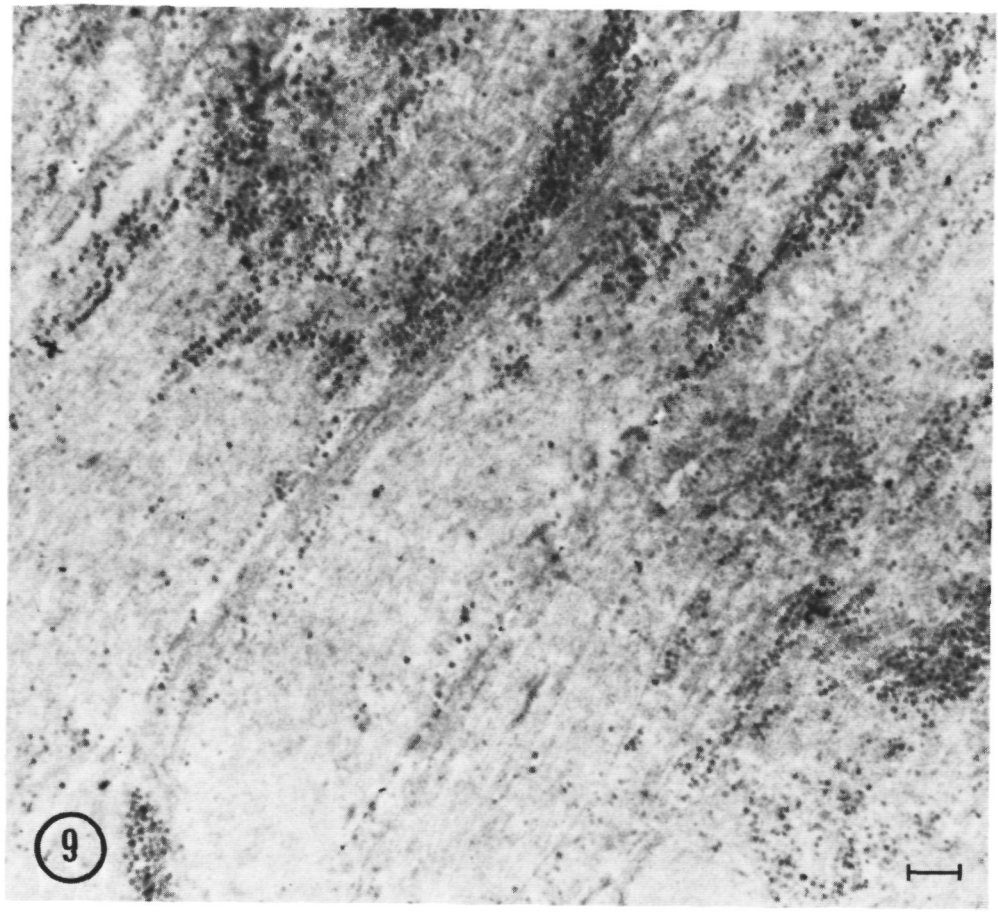
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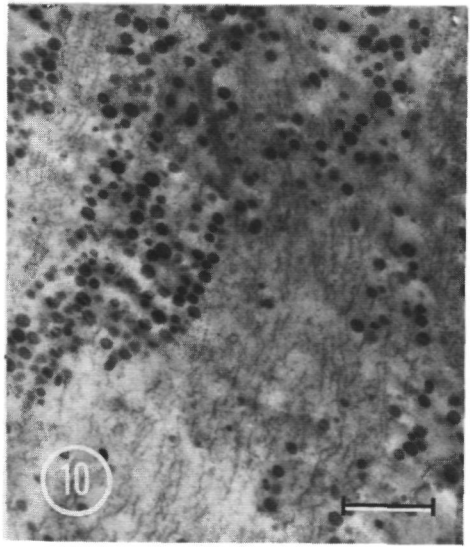
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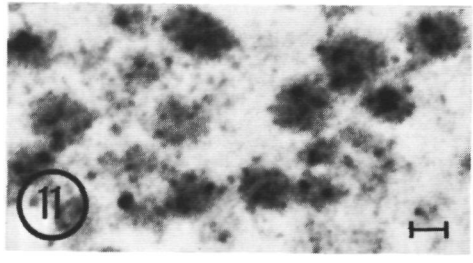
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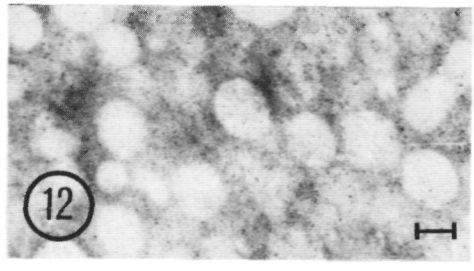
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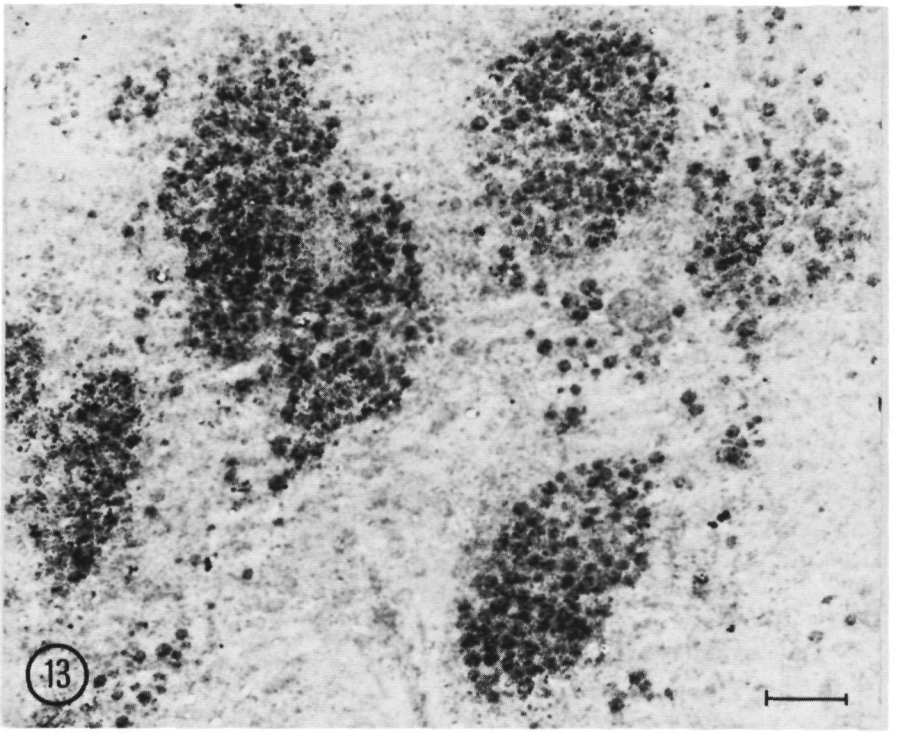
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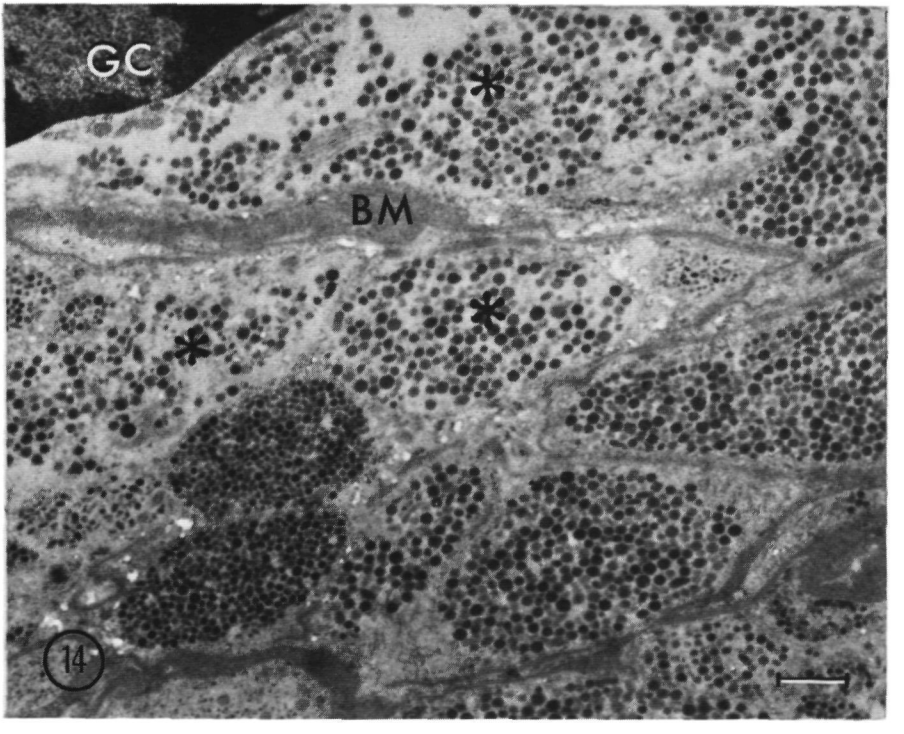
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CHAPTER II

A COMPARATIVE IMMUNOCYTOCHEMICAL INVESTIGATION ON THE LOCATION OF THE CRUSTACEAN HYPERGLYCEMIC HORMONE IN THE EYESTALKS OF SOME DECAPOD CRUSTACEA

Gorgels-Kallen, Van Herp and Leuven (1982),
Journal of Morphology, 174: 161-168.
Presented at the VIIth
Réunion des Carcinologistes de Langue Française,
Banyuls-sur-Mer (01.06-06.06.1981):
Van Herp and Gorgels-Kallen (1981).

SUMMARY

The eyestalks of the astacidean species ***Orconectes limosus***, ***Nephrops norvegicus*** and ***Homarus gammarus***, and the palinuran species ***Palinurus vulgaris***, are investigated immunocytochemically, with an antiserum raised against purified crustacean hyperglycemic hormone (CHH) of the astacidean species ***Astacus leptodactylus***.

A distinct immunopositive reaction can be seen mainly in a group of neurosecretory cells in the medulla terminalis ganglionic X-organ (MTGX), in the MTGX-sinus gland tract and in a considerable part of the sinus gland. The results suggest that the immunoreactive sites in the eyestalk of the investigated species correspond to the site of production, storage and release of the CHH.

Preliminary investigations indicate that a positive immunoreaction can also be obtained in the eyestalk of other decapod crustaceans, for example the brachyuran species ***Macropipus puber*** and the caridean species ***Palaemon serratus*** by use of the above-mentioned antiserum.

INTRODUCTION

Physiological investigations indicate that the neurosecretory cells responsible for the production of the crustacean hyperglycemic hormone (CHH) are generally located in the medulla terminalis ganglionic X-organ (MTGX) and that the CHH is released into the hemolymph via a neurohemal organ, the sinus gland (for a review see Kleinholz and Keller 1979).

Comparative biological and biochemical studies of the CHH in decapod crustaceans furnished evidence for a systematic group specific activity (Keller 1969; Kleinholz and Keller 1973). A disc-electrophoretic study of sinus gland extracts of various species demonstrated marked differences in the electrophoretic mobility of the CHH molecule between the examined groups (Keller 1977). Recently, Leuven et al. (1982) made a comparative biological and immunological study of the occurrence of CHH in several infraorders of the decapod crustaceans. Cross-injection experiments and immunological methods such as micro-double-immunodiffusion and micro-immuno-electrophoresis, with CHH-containing extracts, in combination with anti-*Astacus*-CHH and anti-*Carcinus*-CHH, documented group specificity at the level of superfamilies. No interspecific cross-reactivity was found among the infraorders Astacidea, Brachyura and Caridea. However, these authors indicated that more sensitive methods might give more information about the relationship between specific CHH molecule versions.

As shown in Chapter I, the production of an antiserum raised against the purified CHH-containing fraction of the eyestalk of the crayfish ***Astacus leptodactylus***, allows a precise immunocytochemical determination of the ***Astacus*** CHH-producing system (see also Van Herp and Van Buggenum 1979). Comparable results were obtained for the CHH-producing system of the crab ***Carcinus maenas*** (Jaros 1979; Jaros and Keller 1979) by use of the anti-*Carcinus*-serum.

The aim of the present study is to give a description of the immunopositive sites in the eyestalks of several Astacidea, ***Orconectes limosus***, ***Nephrops norvegicus*** and ***Homarus gammarus***, and a representative of the Palinura, ***Palinurus vulgaris***, by means of the peroxidase-antiperoxidase (PAP) method of Sternberger (1974, 1979), and by use of the anti-*Astacus leptodactylus*-CHH serum. In addition this staining procedure is applied on the eyestalks of ***Macropipus puber*** and ***Palaemon serratus***, representatives of respectively the Brachyura and the Caridea, to test the cross-reactivity between the CHH molecule versions on the immunocytochemical level.

MATERIALS AND METHODS

Animals

Eyestalks of ***Homarus gammarus*** were collected from animals supplied by courtesy of KLM-Catering (Royal Dutch Airline Company). Eyestalks of ***Orconectes limosus*** were obtained from animals collected near the cooling water intake of the electrical power station (PLEM Buggenum) on the river Meuse. Eyestalks of ***Nephrops norvegicus***, ***Palinurus vulgaris***, ***Macropipus puber*** and ***Palaemon serratus*** were studied from animals collected in the bay of Concarneau (France).

Histological and immunocytochemical preparation

Eyestalks were fixed, dehydrated, cleared and embedded in paraplast according to the procedures described in Chapter I.
The general prescription of the immunocytochemical peroxidase-antiperoxidase (PAP) staining procedure, as applied in combination with the anti-***Astacus leptodactylus***-CHH serum, is also given in Chapter I. For testing the optimal dilution of the primary antiserum, a dilution series was made for each investigated species. In conclusion, the sections were incubated overnight at 4°C with the primary antiserum used in diluted form: 1/60 (***Homarus gammarus***), 1/125 (***Nephrops norvegicus***), 1/200 (***Orconectes limosus***), 1/150 (***Palinurus vulgaris***), 1/60 (***Macropipus puber***) and 1/500 (***Palaemon serratus***). The specificity of the immunocytochemical staining was carried out in analogy of Chapter I.

Morphometric analyses

The examined eyestalks of the species ***Orconectes limosus***, ***Homarus gammarus***, ***Nephrops norvegicus*** and ***Palinurus vulgaris*** were analysed morphometrically. Per investigated species (one eyestalk per animal) the number of immunopositive cells were counted and the largest and smallest diameters of the perikarya and the nuclei were determined for a variable number of immunoreactive cells per species. For further details see Table 1.

RESULTS

The immunocytochemical study of the eyestalks of the crayfish ***Orconectes limosus***, the lobsters ***Homarus gammarus*** and ***Nephrops norvegicus***, and the spiny lobster ***Palinurus vulgaris***, with the peroxidase-antiperoxidase (PAP) staining method, reveals a positive reaction to the primary antiserum in a considerable part of the sinus gland, the MTGX-sinus gland tract and a number of neurosecretory cells of the MTGX (Figs 2-9). In all four species, the immunoreactive perikarya form a distinct group, situated rostrally at the most distal part of the MTGX on the latero-ventral region of the medulla terminalis as schematized in Fig 1. Table 1 summarizes the results described below.

The immunopositive neurosecretory cells in the eyestalk of the crayfish ***Orconectes limosus*** form a defined group consisting of about 27 cells. The

largest and the smallest diameters of these perikarya are approximately 56 µm and 33 µm their nuclei measure 15 µm. In the perikarya the immunoreaction is visible as granular material, mainly aggregated into "clumps" distributed at random in the cytoplasm. There is a considerable difference in the intensity of the immunostaining among the immunopositive cells (Fig 2).

In the eyestalk of the lobster ***Homarus gammarus*** the immunopositive cell group consists of approximately 41 cells. The largest and the smallest diameters of the perikarya are about 78 µm and 55 µm and the nuclei measure 15 µm. In the cytoplasm, the immunoreaction is randomally distributed as granular material, but is more dense at the periphery of the cell. A small number of the immunopositive cells shows a slightly less intense immunostaining with the anti-***Astacus***-CHH serum (Fig 4).

As in the above two described species, the immunopositive perikarya in the eyestalk of the lobster ***Nephrops norvegicus*** form a distinct group consisting of some 45 cells. The largest and the smallest diameters of the perikarya are 48 µm and 28 µm, respectively, and their nuclei measure 12 µm. The immunoprecipitate appears as granular material, partly aggregated into "clumps", distributed at random throughout the cytoplasm. In this species there is a great difference in the intensity of the staining among the immmunopositive cells (Fig 6). Besides the above described immunoreactive cells in the MTGX, a single cell in the MEX (medulla externa X-organ), characterized by a perikaryon of 60 µm × 30 µm and a nucleus of 10 µm, also shows an immunopositive reaction; this cell was stainable in half of the examined specimen.

In contrast with all the above-mentioned species, in the eyestalk of the spiny lobster ***Palinurus vulgaris*** the number of positively reacting cells is high (149 cells). The largest and the smallest diameters of the perikarya are 39 µm and 30 µm. The nucleus measures about 13 µm. In the perikarya the immunochemical reaction product has a granular appearance. It is mostly aggregated into "clumps", and is homogeneously distributed over the cytoplasm. Variable intensity of the staining among the immunopositive cells is also present in this species (Fig 8). As in ***Nephrops,*** neurosecretory cells other than those described above in the MTGX show an immunoreaction: three cells in the lamina ganglionaris (perikarya 30 µm × 15 µm, nuclei 8 µm) and about three cells in the MEX (perikarya 30 µm × 15 µm, nucleus 3 µm).

In all the species examined the immunoreaction in the sinus gland is strong and spread over the neurohemal region as aggregated clumps (Figs 3,5,7,9).

A preliminary immunocytochemical study of the eyestalk of a representative species of the brachyuran ***Macropipus puber*** reveals an immunopositive reaction in the sinus gland, in the X-organ-sinus gland tract and in a number of neurosecretory cells in the MTGX and MEX (Figs 10,11). In common with the described astacidean and palinuran species, the immunopositive perikarya in the MTGX form a distinc group situated at the most distal part of the MTGX, rostrally on the latero-ventral side of the medulla terminalis. However, this immunoreaction is very weak and the appearance of the reaction product is not granular but diffuse. Furthermore, it is of some note that the reaction faded a few days after carrying out the PAP-staining in contrast to the reactions in the other crustacean eyestalks.

Immunocytochemical study of the eyestalk of a representative species of the Caridea, ***Palaemon serratus,*** reveals an immunocytochemical reaction in a considerable portion of the sinus gland, in the MTGX-sinus gland tract, and in a number of neurosecretory cells in the MTGX. Also in this species the immunopositive perikarya form a distinct group situated at the most distal part of the MTGX on the latero-ventral part of the medulla terminalis. In their cytoplasm the immunoreaction is present at random and as granular material, mainly aggregated into clumps (Figs 12,13).

The controls of the immunocytochemical staining, tested by substituting buffer in the successive incubations and by substituting normal rabbit serum for the primary antiserum, reveal no immunopositive reaction.

TABLE 1

Quantitative data (means ±S.E.M.) on the number and size of the immunoreactive cells in the eyestalk of three astacidean and a palinuran species.

	Number of eyestalks examined*	Number of immunopositive cells	Number of cells measured	Perikaryon Largest diam. (µm)	Perikaryon Smalliest diam. (µm)	Nuclear diameter (µm)
Orconectes limosus	4	27 (±2)	75	56.5 (±2.2)	33.0 (±2.0)	15.1 (±.0.4)
Homarus gammarus	4	41 (±3)	16	78.0 (±3.3)	55.7 (±1.8)	15.4 (±.0.3)
Nephrops norvegicus	4	45 (±1)	108	48.4 (±1.2)	28.8 (±0.7)	12.0 (±.0.2)
Palinurus vulgaris	3	149 (±11)	446	39.5 (±0.4)	30.3 (±0.3)	13.3 (±.0.1)

* Per animal one eyestalk examined.

DISCUSSION

The present study of the eyestalks of several astacidean species, ***Orconectes limosus***, ***Homarus gammarus*** and ***Nephrops norvegicus*** and a palinuran species ***Palinurus vulgaris***, reveals an immunopositive reaction with the anti-***Astacus***-CHH serum in neurosecretory cells of the MTGX, in the MTGX-sinus gland tract and in a considerable part of the sinus gland, by use of the specific peroxidase-antiperoxidase (PAP) staining method (Sternberger 1974, 1979). Our preliminary results on the eyestalk of a brachyuran species ***Macropipus puber*** and a caridean species ***Palaemon serratus***, point to the same region of location, storage and release of the CHH. In a previous immunocytochemical study, presented in Chapter I, using the same primary antiserum, we localized the hyperglycemic hormone-producing cells in a corresponding region of the eyestalk of ***Astacus leptodactylus*** (see also Van Herp and Van Buggenum 1979). In agreement with those studies are the results of Jaros and Keller (1979) who were able to localize the CHH cells in the eyestalk of the brachyuran species ***Carcinus maenas***, by use of an antiserum raised against extract of entire sinus glands of ***Carcinus maenas***. Recently, an immunocytochemical study on the eyestalks of some Palaemonidae with the same anti-***Astacus***-CHH serum showed a comparable immunoreaction (Van Herp et al., in press). The combination of all these findings indicates that the sites of production, storage and release of the CHH material is comparable in all the infraorders studied: Brachyura, Astacidea, Palinura and Caridea.

In a biological and immunological study concerning species and group specificity, Leuven et al. (1982) report biological cross-reactivity at the level of the infraorders. However, by use of immunological cross-experiments employing the two above-mentioned anti-CHH sera (anti-***Astacus***-CHH and anti-***Carcinus***-CHH), the same authors found no interspecific cross-reactivity among the infraorders Astacidea, Brachyura and Caridea, although cross-reactivity was found on the level of the superfamilies. They concluded that the bioassay seemed to be more suitable for interspecific studies and they indicated that more sensitive methods might give more detailed information about the range of relationships between specific CHH versions. Indeed, in this respect it must be noted that the immunological recognition in the study of Leuven et al. (1982) was based on the formation of an immunoprecipitation reaction in agar gels (immunodiffusion techniques) and that in the present study the PAP technique of Sternberger (1974, 1979) was used. Until now, an immunocytochemical staining has proven to be the most sensitive immunodetection method (Sternberger 1979).

Yet, the eyestalk of ***Macropipus puber*** shows a very weak cross-reactivity with the anti-***Astacus***-CHH serum and the reaction product is only stable for several days after carrying out the immunocytochemical procedure. On the other hand, the eyestalk of the prawn ***Palaemon serratus*** shows an immunoreaction with the anti-***Astacus***-CHH, similar to the Astacidea and Palinura. The possibility that a positive reaction could be caused by the presence of an antigen in the immunocytochemical reacting cells, which coincidentally resembles the immunological reactive sites of the CHH molecule, must be taken in account. However, this possibility seems unlikely because the location of the immunoreactive regions is comparable in all the species investigated. Apparently, there does exist a more or less immunologically detectable relationship between the hyperglycemic hormone of the Astacidea, Palinura, Caridea and Brachyura.

In the immunoreactive perikarya of ***Orconectes limosus***, ***Palinurus vulgaris***, and somewhat less in ***Nephrops norvegicus***, the reaction product is visible as granular material, mainly aggregated into "clumps", comparable to the immunoreaction in the eyestalk of ***Astacus leptodactylus***. The electron microscopic study of the CHH-producing perikarya of ***Astacus leptodactylus*** revealed that

these "clumps" correspond to Golgi zones in the cytoplasm surrounded by immunopositive granules (Chapter I). In the eyestalks of the examined species, except *Homarus gammarus*, there is a great difference in intensity of the immunostaining among the immunoreactive perikarya. In Chapter I we indicated that this difference might be related to different stages in the secretory process and so in differences in amount of antigenic products. These differences in staining intensity have been studied in more detail and are documented in Chapter III. In this respect it is interesting to note that individual synthesis differences have been described earlier by Jaros (1977, 1980b). By use of autoradiographic techniques to investigate RNA and protein synthesis in the type A cells in the eyestalk of *Orconectes limosus*, this author found large individual differences in the levels of both RNA and protein synthesis. The results of Jaros (1977, 1980b) and the present immunocytochemical results indicate that the immunoreactive cells in the MTGX of *Orconectes limosus* are identical to these type A cells. The immunopositive cells in the eyestalk of *Homarus gammarus* show less immunoreaction with the primary antiserum. In the cytoplasm the immunoreactive product is visible as granular, non-aggregated material, which at the periphery of the cell is stained more densely. Among the immunoreactive cells the individual differences in staining intensity are minimal. These phenomena may indicate a rapid transport of the granules into the tract.

It is interesting to consider the difference in the total number of immunopositive cells in the MTGX of the investigated Astacidea and Palinura. This number may be species-depending or related to different physiological conditions such as the rhythm of synthesis-activity of the cells. In this respect, more information has been gathered recently on the CHH-producing system of the prawn *Palaemon serratus*. For this species fluctuations in the number of immunopositive cells have been found during a diurnal and moult cycle (Van Herp et al., in press).

Besides the immunopositive perikarya in the MTGX, sometimes a single cell in the MEX of *Nephrops norvegicus* and some cells in the MEX and the lamina ganglionaris of *Palinurus vulgaris* show an immunopositive reaction with the primary antiserum employed. Jaros (1979) also reports an immunopositive reaction in some cells apart from the MTGX in *Carcinus* which were only stainable in some light microscopic sections. The nature of these immunopositive reactions is dubious. These perikarya might be a site of production of CHH, though this does not seem very likely in the context of the presently available results on CHH. Another explanation might be that these immunopositive perikarya are the site of production of an undetermined protein which is immunocytochemically detectable by the used antiserum.

The method specificity tests, performed as described by Sternberger (1974, 1979), gave no immunopositive results. However, immunostaining after preadsorption of the antiserum with purified CHH of the different species has not been carried out in this study because purified CHH was not available. Also preadsoption with purified *Astacus* CHH was not performed because it should be noted that preadsorption of an antiserum with purified material identical to the antigen used to raise the antiserum, will always eliminate the whole diversity of antibodies that react with this material.

Fig. 1: Schematized location of the CHH-producing cells in the neurosecretory system of the eyestalk of decapod crustaceans. The overall organization was based on the reconstruction of the eyestalk of *Astacus leptodactylus* by J.H.M. Van Deijnen (personal communication).

Fig. 2: Immunocytochemical (PAP) staining of neurosecretory cells in the MTGX of the astacidean species *Orconectes limosus*. Bar represents 50 µm.

Fig. 3: Immunocytochemical (PAP) staining of the tract and the sinus gland of the astacidean species *Orconectes limosus*. Bar represents 100 µm.

Fig. 4: Immunocytochemical (PAP) staining of neurosecretory cells in the MTGX of the astacidean species *Homarus gammarus*. Bar represents 50 µm.

Fig. 5: Immunocytochemical (PAP) staining of the sinus gland of the astacidean species *Homarus gammarus*. Bar represents 100 µm.

Fig. 6: Immunocytochemical (PAP) staining of neurosecretory cells in the MTGX and the proximal part of the tract of the astacidean species *Nephrops norvegicus*. Bar represents 50 µm.

Fig. 7: Immunocytochemical (PAP) staining of the sinus gland of the astacidean s *Nephrops norvegicus*. Bar represents 100 µm.

Fig. 8: Immunocytochemical (PAP) staining of neurosecretory cells in the MTGX of the palinuran species *Palinurus vulgaris*. Bar represents 50 µm.

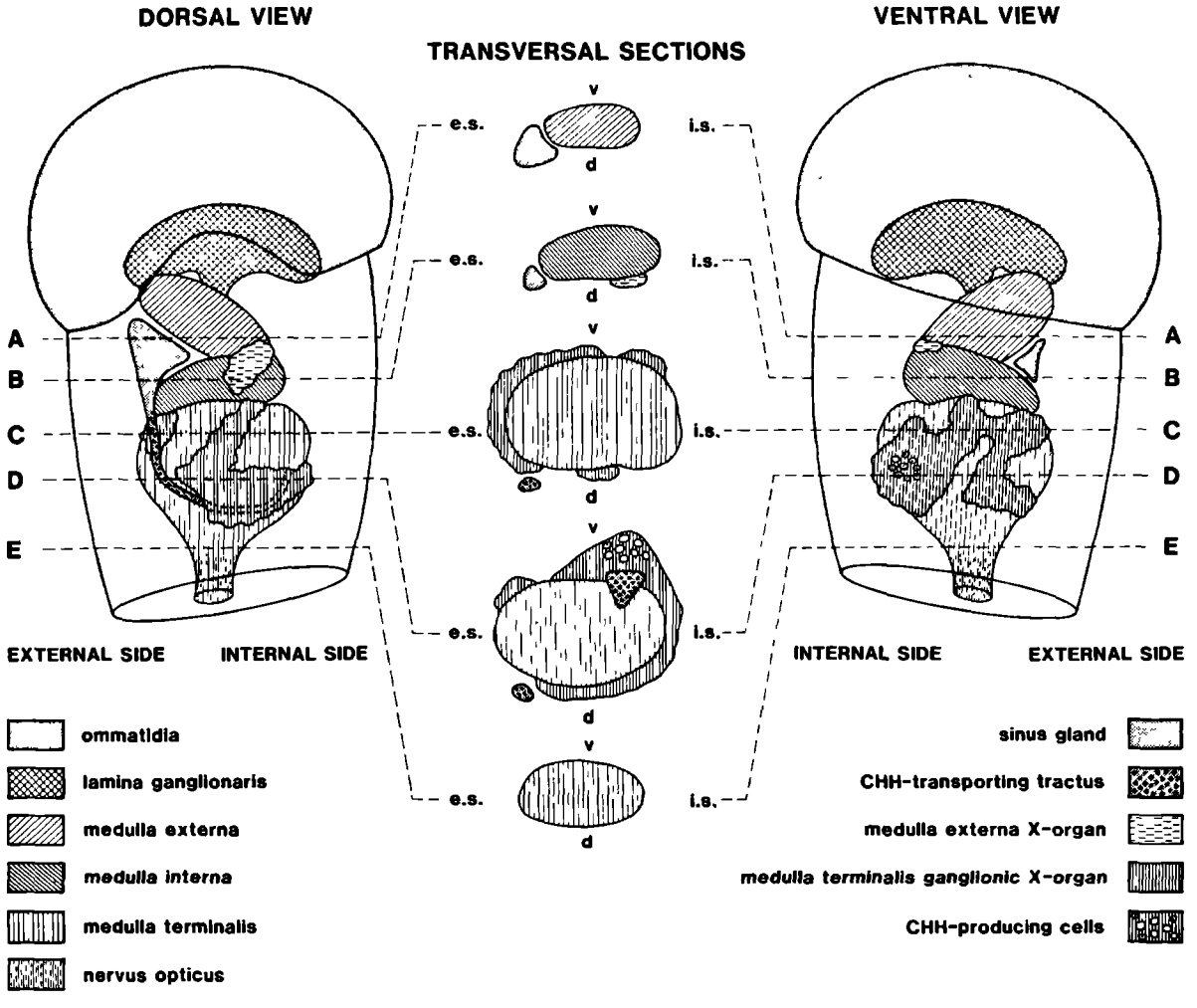
Fig. 9: Immunocytochemical (PAP) staining of the sinus gland of the palinuran species *Palinurus vulgaris*. Bar represents 100 µm.

Fig. 10: Immunocytochemical (PAP) staining of neurosecretory cells in the MTGX of the brachyuran species *Macropipus puber*. Bar represents 50 µm.

Fig. 11: Immunocytochemical (PAP) staining of the sinus gland of the brachyuran species *Macropipus puber*. Bar represents 100 µm.

Fig. 12: Immunocytochemical (PAP) staining of neurosecretory cells in the MTGX of the caridean species *Palaemon serratus*. Bar represents 50 µm.

Fig. 13: Immunocytochemical (PAP) staining of the sinus gland of the caridean species *Palaemon serratus*. Bar represents 100 µm.


DORSAL VIEW
TRANSVERSAL SECTIONS
VENTRAL VIEW
A
B
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E
e.s.
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v
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EXTERNAL SIDE
INTERNAL SIDE
INTERNAL SIDE
EXTERNAL SIDE
ommatidia
lamina ganglionaris
medulla externa
medulla interna
medulla terminalis
nervus opticus
sinus gland
CHH-transporting tractus
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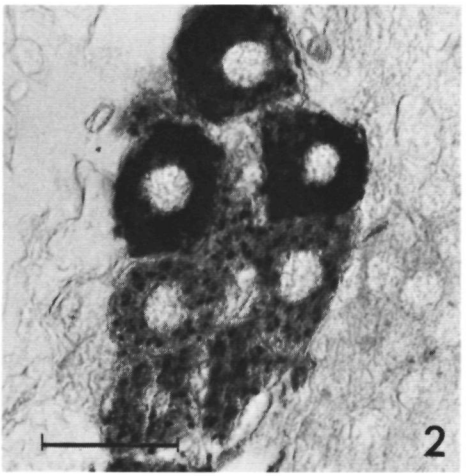
medulla terminalis ganglionic X-organ
CHH-producing cells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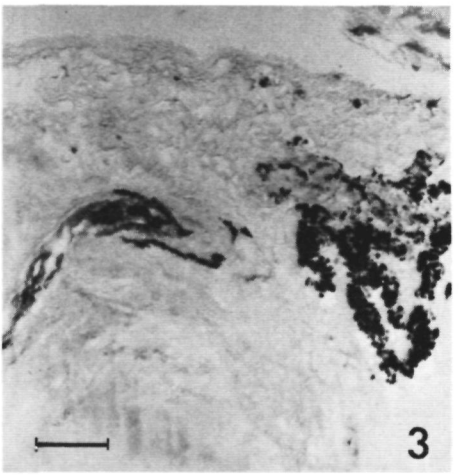
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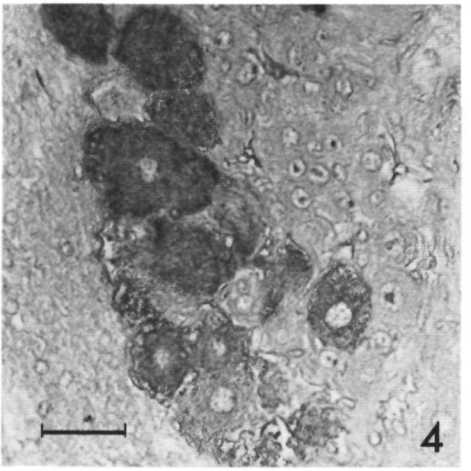
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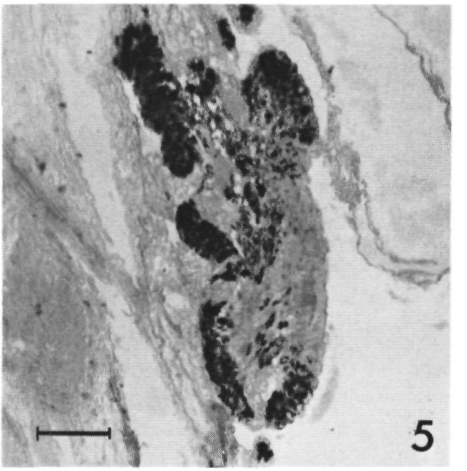
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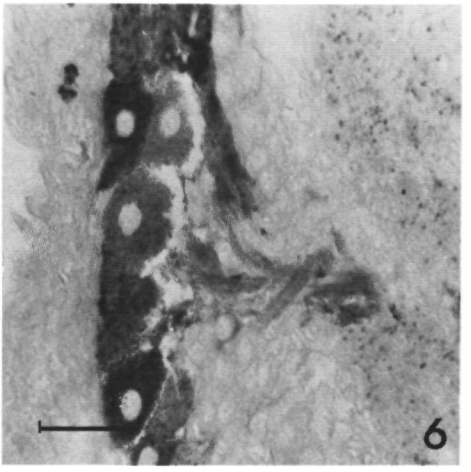
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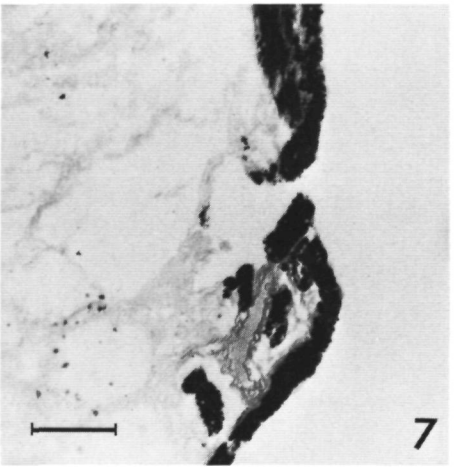
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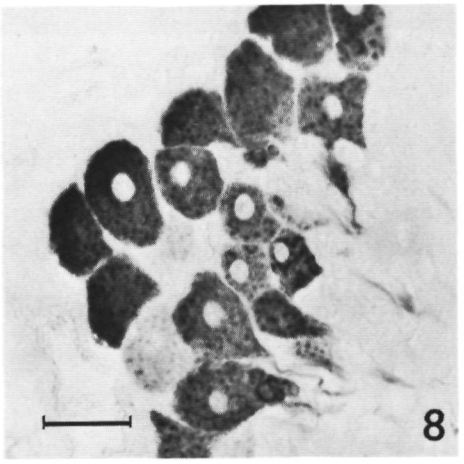
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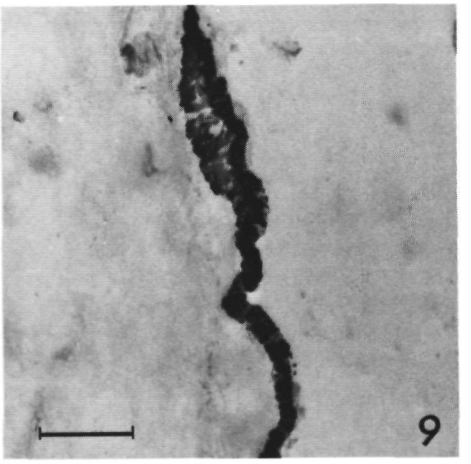
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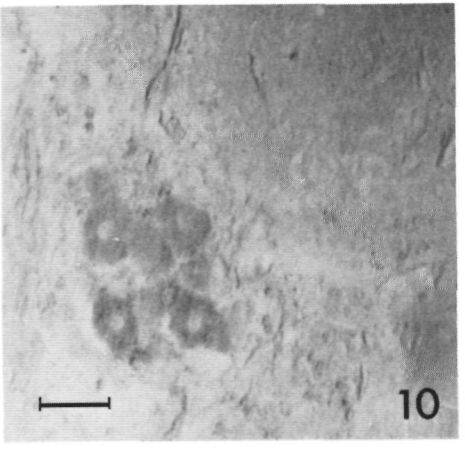
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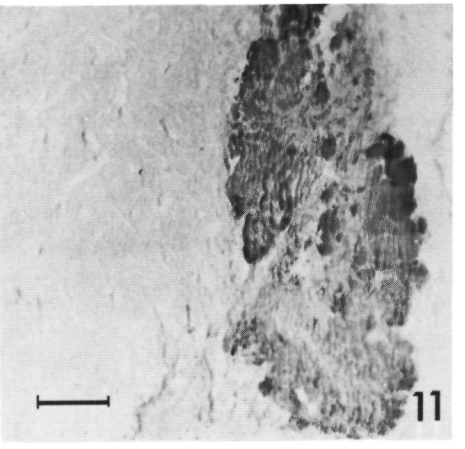
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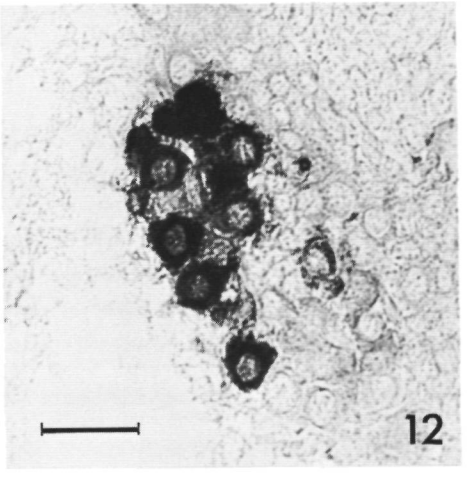
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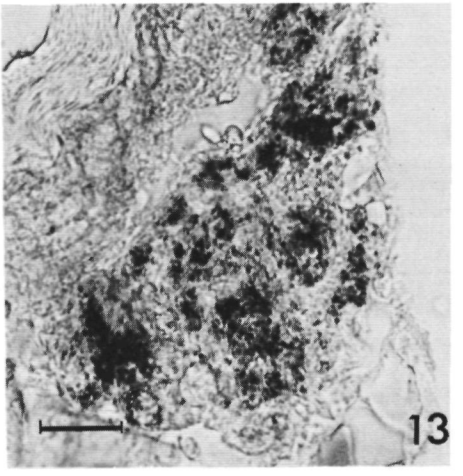
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CHAPTER III

DETERMINATION OF THE SECRETORY STAGES OF INDIVIDUAL CRUSTACEAN HYPERGLYCEMIC HORMONE-PRODUCING CELLS IN THE EYESTALK OF THE CRAYFISH ASTACUS LEPTODACTYLUS, BY AN IMMUNOCYTOCHEMICAL APPROACH

Gorgels-Kallen and Voorter (1984),
Cell and Tissue Research, 237:291-298.
Presented at the VIIIth
Réunion des Carcinologistes de Langue Française,
Liège (29.08-02.09.1983):
Gorgels-Kallen (1984a).

SUMMARY

Immunocytochemical staining demonstrates striking differences in staining intensity between individual crustacean hyperglycemic hormone (CHH)-producing cells in the eyestalk of the crayfish ***Astacus leptodactylus***. Based on these differences we arbitrarily subdivided the CHH cells in three categories representing increasing levels of immunoreactivity: + cells, ++ cells and +++ cells.

Electron microscopic investigations reveal that the differences in immunostaining are correlated with differences in numerical density of the neurosecretory granules in the cytoplasm and that these may reflect differences in activity between the CHH cells. Morphometric analyses at light and electron microscopic levels indeed indicate that the three distinguished categories of immunopositive cells represent different activity levels in the CHH-synthesizing process of the cells.

The results of the present study demonstrate the usefulness of the PAP technique at the light microscopic level as a method to obtain information on the dynamics of secretory activity of the CHH cells.

INTRODUCTION

In Chapter I we reported the immunocytochemical location at the light and electron microscopic levels of the sites of production, storage and release of the hyperglycemic hormone in the eyestalk of the crayfish ***Astacus leptodactylus***, applying the PAP technique in combination with an anti-***Astacus***-CHH serum. The CHH system consists of a group of neurosecretory cells located rostrally at the distal latero-ventral side of the medulla terminalis ganglionic X-organ (MTGX). The secretory granules are transported via an axonal tract to a neurohemal organ, the sinus gland. At the light microscopic level the immunoreactive material in the cell bodies is visible as aggregates scattered in the cytoplasm. At the ultrastructural level these aggregates prove to be Golgi zones surrounded by immunoreactive secretory granules.

Striking features in the above-mentioned studies are the differences in staining intensity among the immunoreactive cells at the light microscopic level and the variation in amounts of immunostainable granules in the cytoplasm of the individual CHH cells at the ultrastructural level. As indicated in Chapter II, variation in intensity of immunostaining among individual CHH cells within one animal was also observed for the CHH system in the eyestalks of several other astacidean and a palinuran species. Comparable results were recently obtained for CHH-producing neuro-endocrine cells in several Palaemonidae (Van Herp et al., in press).

We suggest that these differences in immunoreactivity of the individual cells are correlated to various amounts of antigen in the cytoplasm and so reflect different stages in the secretory process of the cells.

Until now, attempts to collect information on the occurrence of different stages in the secretory process of crustacean neurosecretory cells have not been very successful. At the light microscopic level, differences in the secretory contents of cells in the X-organ or the sinus gland have been described, on the basis of differences in staining affinities of secretory products for various dyes (for a review see Gabe 1966). Ultrastructurally, our knowledge is limited to the work of Shivers (1967) who described different stages of activity of the type 1 neurosecretory cells in the eyestalk of the crayfish ***Orconectes nais***.

Since the introduction of immunocytochemistry, this technique, although mostly used to localize particular products, has proven to be useful as a

semiquantitative method in estimating relative amounts of a certain antigen (e.g. Streefkerk and Van der Ploeg 1973; Moriarty et al. 1973; Sternberger and Petrali 1975; Baron et al. 1978; Benno et al. 1982).
As our anti-CHH serum in combination with immunocytochemistry enabled us to identify the CHH cells and to observe staining differences between these cells, a combined semi-quantitative immunocytochemical and electron microscopic study is undertaken to obtain information on the dynamics of synthetic activity in the CHH-producing cells of the crayfish *Astacus leptodactylus*.

MATERIALS AND METHODS

Animals

Crayfish of the species *Astacus leptodactylus* were obtained commercially and kept in the laboratory as described in Chapter I. Eyestalks and MTGX-organs were collected from normally fed, adult male crayfish in stage C of their moulting cycle (Drach 1944).

Immunocytochemistry

The conventional histological and immunocytochemical procedures for the eyestalks for light microscopy are presented in Chapter I.
On the basis of the differences in intensity of immunoreaction of the individual CHH cells, these were arbitrarily subdivided in three stages: +, ++ and +++, representing the cells with less intense, moderately intense and most intense immunostaining, respectively. To detect any variation in staining intensity due to steric hindrance, as could be caused by high amounts of antigen in the cytoplasm, several dilution series of the primary antiserum were used and the incubation time was varied.

For a combined light and electron microscopic study, eyestalk material was prepared as follows. The group of CHH cells (for details on the location of the CHH cells see Chapter II) was dissected from the MTGX and fixed in 2% glutaraldehyde buffered with 0.1 M sodium-cacodylate (pH 8; containing 0.2 M sucrose), for 45 min on melting ice. Postfixation followed in 1% osmiumoxide in the same buffer, for 1 h also on melting ice. After rinsing in 50% acetone, the tissue was block-stained with 1% uranyl acetate in 50% acetone for 30 min. The specimens were dehydrated in a graded acetone series and embedded in Spurr's resin (Spurr 1969). The tissue was sectioned serially, in a sequence of ±10 semithin sections (1.5 µm) followed by ±30 ultrathin sections.
The semithin sections were mounted on glass slides and prepared for immunostaining adopted to the method of Lane and Europe (1965) and Baskin et al. (1979). To remove the plastic, the sections were etched in a saturated solution of sodium hydroxide in 100% ethanol for 45 min, followed by 3 short rinses in 100% ethanol. The sections were hydrated in a graded ethanol series and rinsed in distilled water. After bleaching in 1% periodic acid for 5 min and rinsing in distilled water, the sections were equilibrated in Tris-HCl buffered saline (pH 7.6). PAP staining was accomplished as described for light microscopy (Chapter I), with slight modifications: a. the sections were incubated with solutions of primary antiserum, GAR and PAP-complex in Tris-HCl buffered saline containing 1% NGS (Baskin et al. 1979); b. 3,3'-Diaminobenzidine-4HCL (DAB)/peroxide was used as the final peroxidase substrate.

The ultrathin sections were mounted on formvar/carbon coated single slot grids or uncoated 150 mesh grids and poststained with 2% aqueous uranyl acetate and lead citrate (Reynolds 1963).

Morphometric analyses

The morphometric results of the light microscopic study were based on the analyses of 38 eyestalks from different animals. For each eyestalk the number of +, ++ and +++ cells and the volumes from the perikaryon and nucleus of 5 +, 5 ++ and 5 +++ cells were determined as follows.
Profiles of successive sections from each cell were drawn at a microscopic magnification of 850× by use of a drawing prism. Per examined cell the areas of all sections were measured by Hardware Kontron MOB equipment (Kontron Messgeràte GmbH). Per cell both the protoplasmic and the nuclear volumes were determined by multiplying the total area of all sections from one cell with the thickness of the sections (7 µm) method described by Weibel (1979). For quantitative electron microscopy of the CHH cells, groups of cells from 2 animals were examined. The immunostained semithin sections served to indentify the CHH cells in the ultrathin sections. The number of +, ++ and +++ cells and the protoplasmic and nuclear volumes of the Spurr-embedded CHH cells were also determined in semithin sections. For the electron microscopic observations a Philips 201 or 300 electron microscope was used at 60 kV, and calibrated with a carbon replica grid (2160 lines/mm). For each CHH cell group examined, 3 +, 3 ++ and 3 +++ cells were investigated in the following way: micrographs (final magnification 26000×) were taken from the total surface of a cross-section through the middle of the cell body that included the nucleus, respectively a cross-section taken at the periphery of the cell body where a profile of the nucleus was absent. The number and surface area of the Golgi zones, mitochondria, lysosomal bodies and granules were determined per 1000 µm² surface area of cytoplasm by means of Kontron MOB equipment (Kontron Messgerate GmbH). The volumes of the different organelles per cell volume were calculated by the method of Weibel (1979). The data were statistically analyzed with the Student-t-test (two sided; significance accepted when $P<0.05$). The activity level of the observed Golgi zones was estimated by determining the ratio of "active" and "inactive" Golgi zones as described by Wendelaar Bonga (1971).

RESULTS

Light microscopic observations

Immunostaining with an anti-*Astacus*-CHH serum of the CHH-producing system in the eyestalk of *Astacus leptodactylus* reveals striking differences in staining intensity among the individual cells. With the light microscope, the +, ++ and +++ cells are easily distinguishable by the amount and distribution of the immunostainable material in the cytoplasm (Figs 1,2).
In conventional paraplast embedded sections the cytoplasm of the + cells shows weakly stained, randomly distributed granular material, while in the cytoplasm of the ++ cells the granular material is mainly aggregated into evenly distributed and intensely stained "clumps". The cytoplasm of the +++ cells contains deeply stained aggregated clumps of granular material, that mostly

fill up the whole cytoplasmic area. It must be noted, however, that the subdivision of the CHH cells in +, ++ and +++ cells is arbitrary and that a few cells show sometimes a staining pattern that is intermediate between these three cell stages. In the plastic embedded semithin sections, as illustrated in Fig 2, the differences in immunostaining are less pronounced than in paraplast sections, which is likely due to the reduced section thickness in combination with the osmium fixation.

For all incubations an overnight incubation with a 1/150 dilution of the anti-CHH serum at 4°C gave an optimal immunostaining. Furthermore, the results of the dilution series reveal that the intensity of the immunostaining decreases simultaneously in the three cell stages corresponding with incubations of more diluted anti-CHH serum. Increase in immunostaining of individual cells corresponding with increasing dilution of the primary antiserum is not observed: the number of +, ++ and +++ cells does not change throughout the dilution series.

The incubations performed to test the specificity of the immunoreaction reveal no immunopositive results.

Morphometric analyses

To elucidate the factors responsible for the above-mentioned differences in staining intensity of the CHH cells, a variety of morphometric parameters was studied at the light (Table 1) and electron microscopic level (Table 2).

At the light microscopic level differences are evident in the cytoplasmic and nuclear volumes of the +, ++ and +++ cells. The volume of the ++ cells is largest and the volume of the +++ cells is smallest. As for the nuclear volume, the +++ nucleus is smallest. Morphometric analyses of semithin Spurr-embedded sections reveal the same ratio of the numbers of +, ++ and +++ cells, and their cytoplasmic and nuclear volumes are similar with the paraplast-embedded 7 µm sections. By determining the location of the individual cells in the immunostained semithin sections with the light microscope, individual CHH cell stages could be detected on the subsequent ultrathin sections.

At the ultrastructural level marked differences are apparent in the distribution, numerical density, surface area and structure of the neurosecretory granules between the three cell stages. The lowest number of granules is found in the + cells; they appear electron dense and their mean diameter is smallest. Most of the present granules in the + cells are spread randomly over the cytoplasm with a slight accumulation around the Golgi zones (Fig 3A).

In the cytoplasm of the ++ cells the number of granules is higher than in the + cells and they are mostly accumulated around the Golgi zones (Fig 3B). The mean diameter of the granules has increased significantly if compared to the + cells. Besides, in the ++ cells, the mean diameter from the granules increases from the region around the nucleus (±122 nm) to the periphery of the cell (±130 nm).

The highest number of granules is found in the +++ cells. They form not only more extended accumulations around the Golgi complexes but also at the periphery of the cell (Fig 3C). Furthermore, the electron density of a part of the granules in the +++ cells is decreased if compared to the granules of the + and ++ cells (Figs 3C, 5). If we consider the marked differences in total volume between the three cell stages, the difference in total granular content per cell is even more pronounced: around 330 μm^3 in the + cells, around 550 μm^3 in the ++ cells and around 850 μm^3 in the +++ cells.

By studying individual micrographs, no differences in surface area or numerical density of Golgi zones and mitochondria can be distinguished between the

three cell stages. However, if we take into account the differences in total cytoplasmic volume between the three cell stages, we may conclude that in the entire cell bodies marked differences occur for the above-mentioned organelles: per cell the calculated mean of the total Golgi zone volume varies from 730 μm^3 in the + cells and 970 μm^3 in the ++ cells to around 850 μm^3 in the +++ cells; the respective values for the mitochondria are 1500 μm^3 in the + cells, around 1950 μm^3 in the ++ cells and approximatively 1370 μm^3 in the +++ cells.

No differences could be distinguished in the synthetic activity of the Golgi complexes between the three cell stages. All Golgi zones examined show signs of secretory activity like membranes containing electron dense neurosecretory material and/or secretory granules in the phase of budding off from the Golgi membranes (Fig 4).

Rough endoplasmic reticulum (RER) is abundant throughout the cytoplasm of all three cell stages and occurs in vesicular form rather than as extensive arrays (Fig 5). Because of the random location of the RER among the other cellorganelles, it was not possible to make a reliable measurement.

Free ribosomes are common and are mostly located in clusters (polyribosomes), as indicated with arrows in Fig 5.

At the periphery of the cytoplasm, occasionally large vacuoles (±1 µm in diameter) are observed (Fig. 8).

Furthermore, in the +++ cells the number of lysosome-like bodies is significantly higher than in the + and ++ cells.

No differences are found in the distribution of the cellorganelles mentioned throughout the cytoplasm. This is concluded from examination of sections of cells through the middle of the cell including the nucleus and sections taken at the periphery of the cell body. The only exceptions are the unequal distribution of the secretory granules and the occasionally observed vacuoles, as reported above.

It can be noted that the CHH-producing cells are isolated from each other by means of a single or multi-lamellated sheath composed of glial cytoplasmic processes, that often form trophospongia (Fig 6). Occasionally, a lysosome-like body was found in the glial cytoplasm directly adjacent to the CHH perikaryon (Fig 7).

TABLE 1

Morphometric data of the CHH-producing neurosecretory cells of the crayfish ***Astacus leptodactylus***, obtained with light microscopy (means ±S.E.M.).

cell stage	+	++	+++
number of cells (n=38)	9 (±1)	19 (±1)	9 (±1)
cell volume (μm^3) (n*=180)	32,500 (±70)^s	38,800 (±70)^s	27,800 (±70)^s
nuclear volume (μm^3) (n*=180)	5,500 (±10)	5,600 (±10)	4,100 (±10)^s

n = number of animals investigated; per animal one eyestalk was counted.
n* = number of cells examined; per eyestalk ±5 cells of each stage were counted.
s = significantly different from both other groups; Student-t-test (two sided, $P<0.001$).

TABLE 2

Morphometric data on the CHH-producing neurosecretory cells of the crayfish ***Astacus leptodactylus***, obtained by electron microscopy (means ±S.E.M.).

cell stage	+ (12)*	++ (10)*	+++ (12)*
number of granules	1,046 (±111)	1,260 (±203)	2,664 (±442)
area of granules (μm^2)	12 (±1)^s	16 (±2)^s	34 (±5)^s
diameter of granules (nm)	122 (±0.6)^s	127 (±0.6)^s	132 (±1)^s
number of Golgi zones	16 (±3)	12 (±1)	15 (±3)
area of Golgi zones (μm^2)	27 (±4)	28 (±6)	26 (±3)
number of mitochondria	400 (±32)	397 (±28)	382 (±33)
area of mitochondria (μm^2)	54 (±4)	59 (±3)	58 (±3)
number of lysosome-like bodies	9 (±3)	7 (±2)	19 (±3)^s
area of lysosome-like bodies (μm^2)	2 (±0.4)	1 (±0.4)	3 (±1)

Two animals were investigated (one MTGX-region per animal). *The total number of investigated cells is indicated in parentheses. Number and area per 1,000 μm^2 cytoplasm. s = significantly different from both other groups; Student-t-test (two sided, $P<0.001$).

After PAP-staining differences in intensity of the immunoreaction of individual CHH cells become evident. Based on these differences, we arbitrarely subdivide the immunopositive CHH cells in three categories of cells with increasing immunoreactivity, namely +, ++ and +++. After diluting the primary antiserum, a simultaneous decrease in staining intensity in the three stages appeares. The staining diminishes with increasing dilution of the anti-CHH serum. This suggests that the difference in staining intensity between individual CHH cells is due to differences in the amount of antigen present in the cytoplasm. Steric hindrance for the immunochemical reaction does not interfere with the stainability of the cells. So, the amount of antigen is the lowest in the + cells and the highest in the +++ cells.
The present ultrastructural analyses show that the numerical density of the granules is positively correlated to the intensity of the immunostaining. In Chapter I we reported that after PAP-staining of the CHH-producing system at the ultrastructural level the immunopositive reaction is limited to the neurosecretory material in the Golgi zones and the secretory granules. Therefore, we conclude from the present observations that the differences in intensity of the immunostaining are primarily caused by varying numbers of secretory granules in the cytoplasm.

We observed that the mean diameter of the neurosecretory granules is different in the three cell stages and that the increase in diameter is accompanied by a decrease in electron density of the granules, which is most evident in the +++ cells. In Chapter I we noted differences in the diameter of the immunopositive CHH-containing granules between the perikaryon (130 nm), the tract (138 nm) and the sinus gland (143 nm), which implies a growth of the CHH granules following their formation in the perikaryon and transport to the sites of release in the axon terminals. Our observation, that growth of the CHH granules is associated with a decrease in electron density of the granules, is in line with the results of Strolenberg et al. (1977b). These authors studied the sinus gland in *Astacus leptodactylus* and described the granule type V, by us identified as the CHH granule, as moderately electron dense. We conclude that the CHH granules are growing steadily and decrease in electron density after their formation in the Golgi area. The observed differences in granule diameter and electron density between the three cell stages indicate therefore that the secretory granules are youngest in the + cells and oldest in the +++ cells.
Differences in diameter and electron density have been described for neurosecretory granules from vertebrates and from many invertebrate groups (Berlind 1977). These differences in the appearance of secretory granules have frequently been interpreted as reflecting biochemical changes in the nature of the granule content. This process is generally referred as maturation of the elementary granules (e.g. Gerschenfeld et al. 1960; Zambrano and De Robertis 1967; Wendelaar Bonga 1970; Cannata and Morris 1973; Smith 1975; Berlind 1977; Van Minnen and Reichelt 1980). The changes we observed in diameter and electron density of the CHH granules may also be interpreted as the morphological evidence for biochemical changes in the nature of the hyperglycemic material. In Chapter VIII more information is given on the occurrence of molecular heterogeneity of the hyperglycemic hormone present in the neurosecretory system of *Astacus leptodactylus*. Biochemical changes in the CHH granule content might result in changes in affinity for the primary antiserum, which might cause the observed differences in staining

intensity of the immunoreaction in individual CHH cells. However, in our studies we never observed differences in affinity for the primary antiserum between granules in the perikaryon, the tract or the sinus gland.

We observed considerable differences in cytoplasmic and nuclear volumes between the three stages of the CHH cells: they are smallest for the +++ cells and largest for the ++ cells. Although there are no marked differences in numerical density for the Golgi complexes and the mitochondria between the three cell stages, the total volume per cell of these cell organelles is in accordance with the differences in cytoplasmic volumes: lowest in the +++ cells and largest in the ++ cells. All the above-mentioned differences can be considered as indications for variations in secretory activity (Berlind 1977). In this respect, we support that the activity level is lowest in the +++ cells and highest in the ++ cells.

As has been established for many neurosecretory cells, the Golgi apparatus is involved in the formation of neurosecretory elementary granules (for a review see Whaley and Dauwalder 1979). All Golgi complexes examined in the three cell stages apparently were actively synthesizing since all Golgi profiles contained electron dense neurosecretory material and/or were budding off elementary granules. Changes of secretory functions have frequently been related to an increase or decrease in "active" Golgi zones. However, changes in activity level of Golgi complexes may also be reflected by changes of their volume (e.g. Pilgrim 1969). Therefore, we presume that CHH production continues during the whole activity cycle of the individual cells, while the level of secretory activity is determined by alterations in volume of the Golgi zones. Yet, the possibility remains that there are CHH cells which contain no secretory material. Such cells escape detection by means of the presented approach, as they will not show an immunopositive reaction. However, this last possibility will not be very likely because the totai number of immunopositive cells per eyestalk appeared to be constant during different physiological conditions (Chapters IV and V).

The present study shows that different stages in the CHH-synthesizing process are distinguishable. The data obtained point all in the same direction; the suggested relationship between the ultrastructure and the synthetic activity of the CHH cells is summarized in the diagram of Fig 9. The content of CHH in the + cells is low, but the cells have a large nucleus which points to an increased level of synthetic activity by means of mRNA production. The ++ cells represent a cell stage where the cells are more active in producing neurosecretory material than in stage +. In their cytoplasm the number of granules, for the majority located near the Golgi zones, has increased. The synthetic level of the +++ cells is low; they possess the smallest nuclear and cytoplasmic volumes. The number of CHH-containing granules has increased further. Apparently the CHH granules are stored in the cytoplasm before being transported to the axon terminals. The inverse relationship between the amount of secretory material and the synthetic activity has also been reported for other neurosecretory cells (Berlind 1977). The majority of the granules in the +++ cells is located near the periphery of the cell. These granules show a reduced electron dense core and an increased diameter. Both observations point to the occurrence of a maturation process.

Finally, the present study demonstrates that the PAP technique at the light microscopic level can yield information pertaining the dynamics of the content of antigen in the cytoplasm, without employing an extensive morphometric study with the electron microscope. This opens the possibility to study the synthetic dynamics of the CHH system under different physiological conditions like the day/night cycle (Chapter IV) and the moult cycle (Chapter V).

ABBREVIATIONS

CHH	CHH cell
G	Golgi zone
GL	glial cytoplasm
L	lysosome-like body
M	mitochondrium
N	nucleus
T	trophospongium
V	vacuole

LEGENDS

Fig. 1: PAP staining of a 7 µm paraplast embedded section, illustrating the appearance of the +, ++ and +++ cells. Bar represents 50 µm.

Fig. 2: PAP staining of a semithin Spurr embedded section, illustrating the appearance of the +, ++ and +++ cells. Bar represents 50 µm.

Fig. 3A: Ultrathin section of part of the cytoplasm of a + cell. Arrows indicate neurosecretory granules. Bar represents 1 µm.

Fig. 3B: Ultrathin section of a ++ cell. Note the increased number of granules around the Golgi zones. Bar represents 1 µm.

Fig. 3C: Ultrathin section of a +++ cell. Note the accumulations of granules around the Golgi zones and at the periphery of the cell. Bar represents 1 µm.

Fig. 4: Picture of an actively secreting Golgi complex. Bar represents 1 µm.

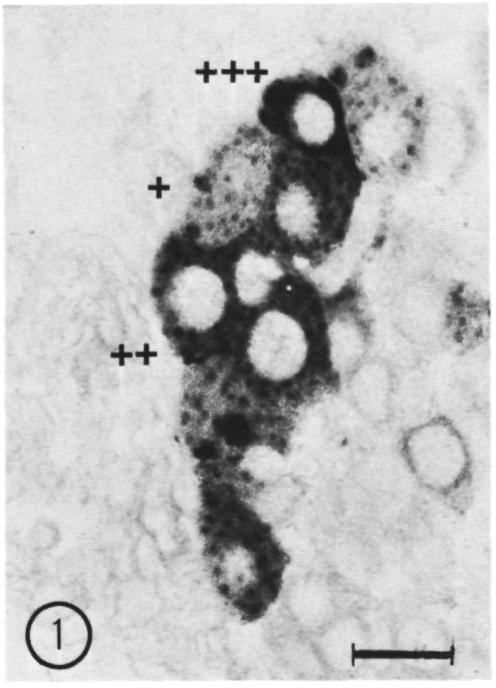
Fig. 5: Illustration of the differences in electron density of the granules in the +++ cell. The RER appears in vesicular form. Ribosomes are clustered together forming polysomes (arrow). Bar represents 1 µm.

Fig. 6: Illustration of the glial cytoplasmic processes surrounding the CHH cells, forming trophospongia. Bar represents 1 µm.

Fig. 7: Lysosome-like body in the glial cytoplasm adjacent to a CHH cell. Bar represents 1 µm.

Fig. 8: Vacuole in the cytoplasm of a CHH cell. Bar represents 1 µm.

Fig. 9: Diagram illustrating the proposed synthesis cycle of the CHH-producing cells of *Astacus leptodactylus*.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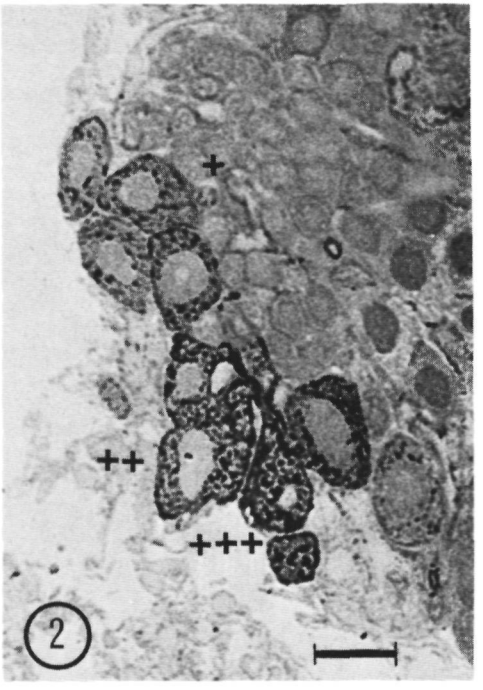
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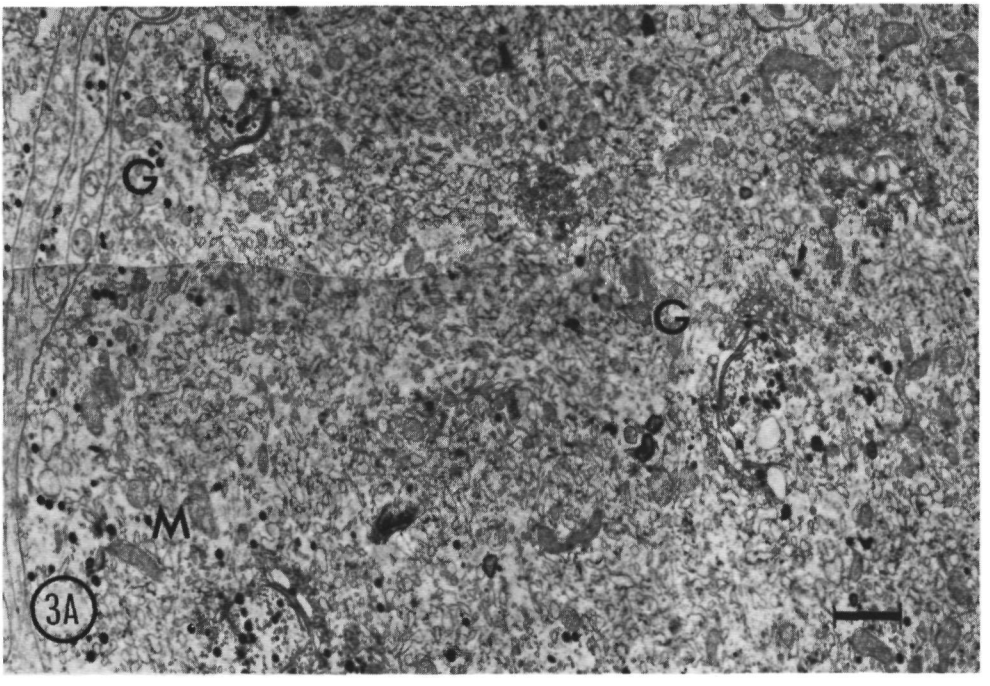
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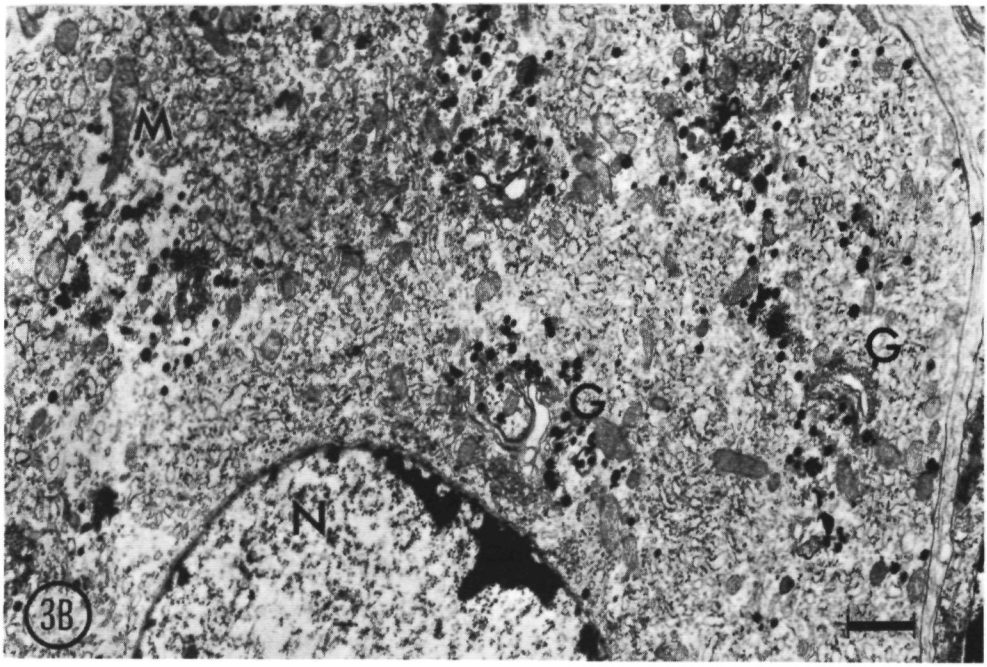
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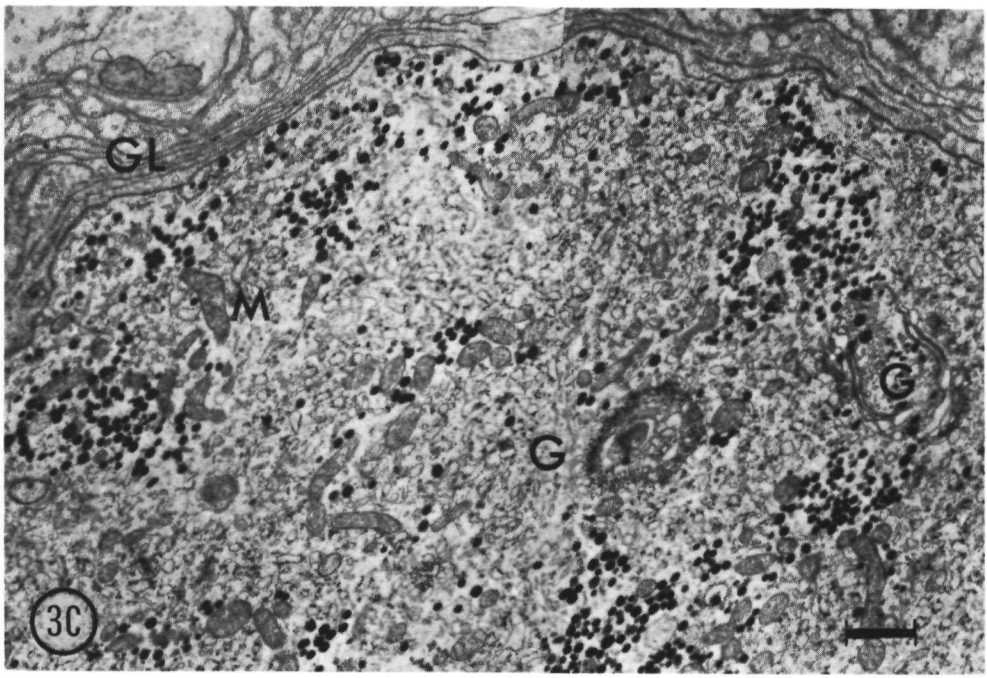
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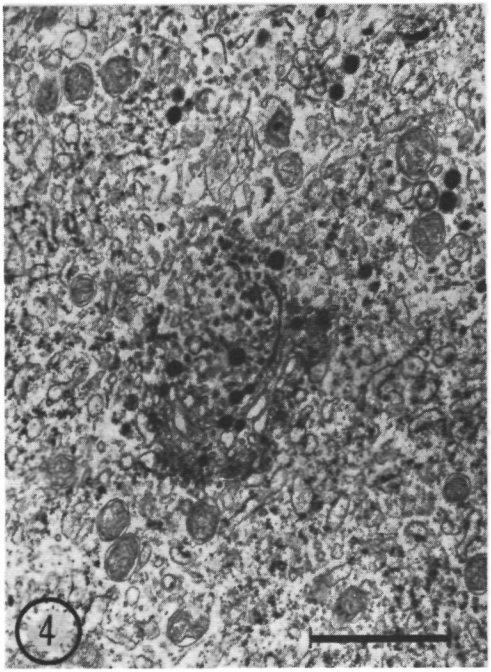
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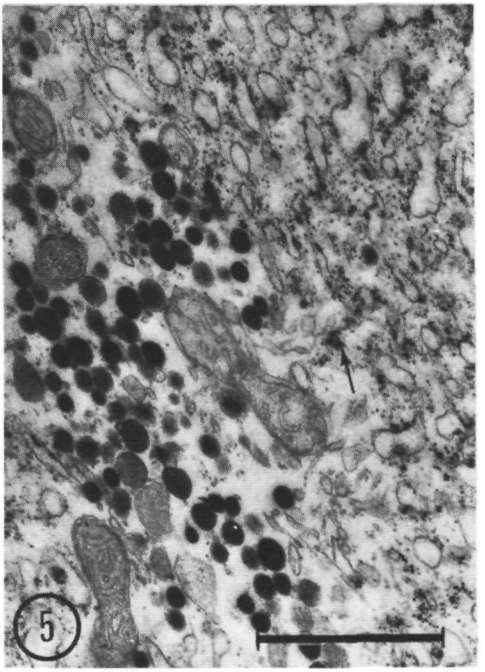
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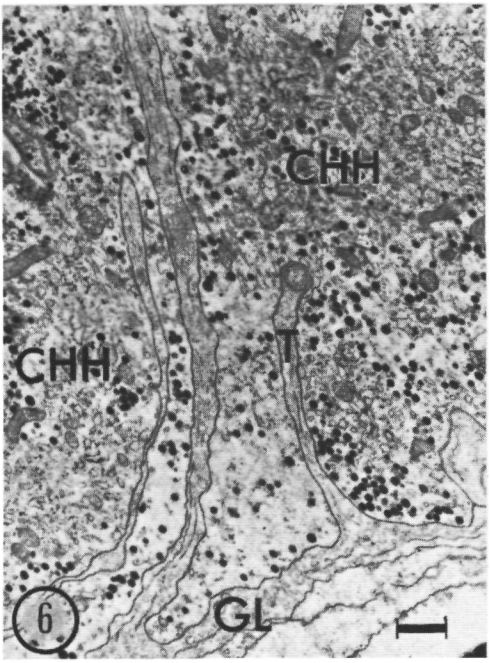
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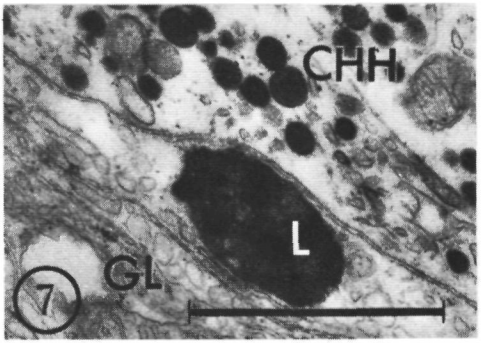
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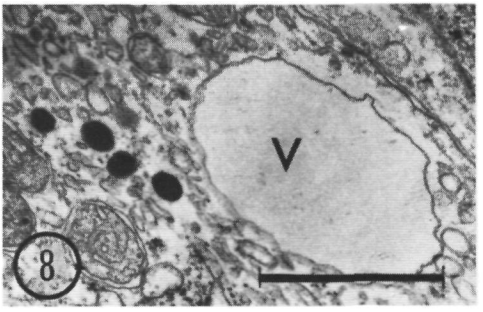
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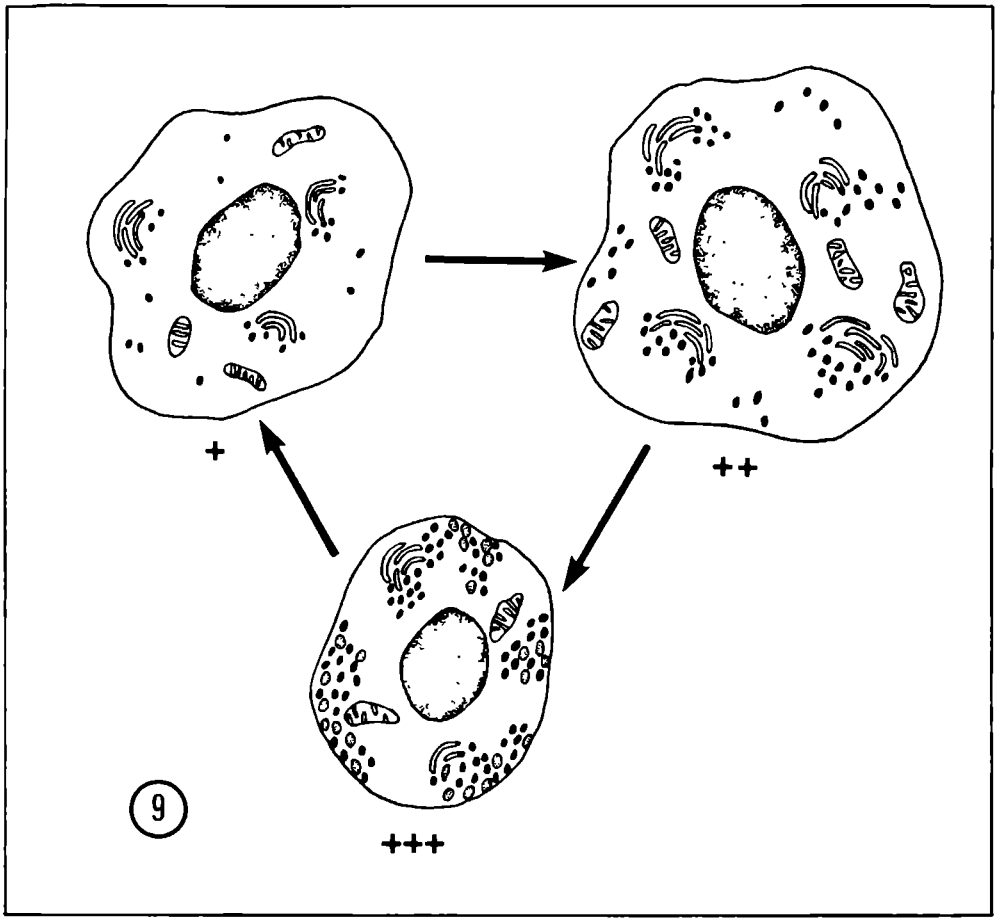
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CHAPTER IV

THE SECRETORY DYNAMICS OF THE CRUSTACEAN HYPERGLYCEMIC HORMONE-PRODUCING CELL GROUP IN THE EYESTALK OF THE CRAYFISH ASTACUS LEPTODACTYLUS, IN THE COURSE OF A DAY/NIGHT CYCLE

Gorgels-Kallen and Voorter,
Cell and Tissue Research, submitted.
Presented at the meeting of the
Dutch British Endocrine Society, Noordwijkerhout,
August 1982, Gorgels-Kallen and Van Herp (1982).

SUMMARY

The secretory dynamics of the crustacean hyperglycemic hormone (CHH)-producing cells in the eyestalk of the crayfish ***Astacus leptodactylus***, were studied during a day/night period (12 h light/12 h dark). The different secretory stages of the individual cells were determined by means of immunocytochemistry combined with morphometric analyses at the light microscopic level. The data obtained were correlated with the 24 h rhythmicity of the blood glucose concentration.

The obtained data support the following hypothesis. The synthetic activity of the CHH cells is stimulated 2 h before the dark period, resulting in a pronounced transfer of CHH granules into the axons. These CHH granules apparently reach the axon terminals at the onset of the dark period. At that time a burst of exocytotic activity follows, causing a strong release of CHH into the hemolymph. This is followed by marked hyperglycemia four hours later.

INTRODUCTION

In Chapter III we describe different stages in synthetic activity of the CHH cells of ***Astacus leptodactylus***, on the basis of differences in density of the individual cells after immunostaining. We arbitrarily subdivided the CHH cells in +, ++ and +++ cells, respectively, corresponding with increasing immunoreactivity as observed light microscopically. The ultrastructural and morphometric data obtained, revealed that the intensity of the immunoreaction depends on the number of neurosecretory granules in the cytoplasm. Moreover, firm differences were found in the cytoplasmic and nuclear volumes of the above-mentioned cell stages. These results led to the conclusion that the three distinguished categories of immunopositive cells represent different stages in the CHH-synthesizing process of the cells.

Hamann (1974) described a circadian rhythm in the glucose content of the hemolymph of the crayfish ***Orconectes limosus:*** the glucose level reaches a peak during the night period. A similar circadian blood glucose rhythm was found for ***Astacus leptodactylus*** (Strolenberg 1979) and for the field crab ***Oziotelphusa senex senex*** (Reddy et al. 1981). As previous investigators already demonstrated that the glucose level in the hemolymph is hormonally regulated (for a review see Kleinholz and Keller 1979), we assume a direct relationship between the production and release of CHH and the observed rhythmicity in the hemolymph glucose level. The main objective of this study is to investigate this hypothesis.

We studied the dynamics of the secretory activity of the CHH cells during a 24 h period (12 h light/12 h dark), based on the description concerning the secretory activity stages of the individual cells presented in Chapter III. In addition, the accumulation (packing) of the granules in these terminals was investigated, in order to gain more information concerning the rhythmicity of release and the new supply of granules from the perikarya.

Animals

As described in Chapter I, crayfish of the species ***Astacus leptodactylus*** were obtained commercially and kept in the laboratory under constant conditions of 12 h light (8.00-20.00) and 12 h dark (20.00-8.00). Blood samples, eyestalks and sinus glands from adult male test-animals of equal size and in intermoult stage (Drach 1944) were collected after a starvation period of at least three days and acclimatization to the laboratory conditions for approximatively 3 weeks. The experiments were performed in July.

Light microscopy

Left eyestalks were collected every 2 hours during a 24 h period. Fixation, dehydration, embedding in paraplast and the immunocytochemical staining procedure for the 7 µm sections were performed according to the methods described in Chapter I.

Electron microscopy

Right sinus glands were collected every 2 hours, except for the period 20.00-24.00 h, when hourly samples were taken. The tissue was fixed in ice-chilled 2.5% glutaraldehyde, buffered with 0.1 M sodium cacodylate (pH 8; 0.2 M sucrose) for 2 h. Postfixation for 1 h followed in ice-chilled 1% osmiumoxide in the same buffer. After dehydration in ethanol, embedding was carried out in a mixture based on Epon 812 (Luft 1961). Ultrathin sections were poststained with 2% aqueous uranyl acetate and lead citrate (Reynolds 1963). Sections were examined in a Philips 300 electron microscope at 60 kV, which was calibrated by use of a carbon replica grating grid (2160 lines per mm).

Morphometric analyses at the light microscopic level

The secretory stages of the CHH cells were determined on the basis of differences in intensity of the immunoreaction as described in Chapter III. Per sample time, the numbers of +, ++ and +++ cells were counted in the left eyestalk from 3 animals. In addition the cytoplasmic and nuclear volumes of 5 +, 5 ++ and 5 +++ cells per eyestalk were calculated after the method of Weibel (1979), as described in Chapter III. The data were statistically analysed by use of the Student-t-test(two sided; significance accepted when $P<0.05$).

Morphometric analyses at the electron microscopic level

Per sample time one sinus gland of each of 5 animals was analysed. The packing of the CHH granules (i.e. the number of neurosecretory granules per unit of surface) was determined by use of Hardware Kontron MOB equipment (Kontron Messgeräte GmbH) and micrographs with a final magnification of

42320×. The data were statistically analysed by the Student-t-test (two sided; significance accepted when $P<0.05$).

Determination of the hemolymph glucose level

The glucose level of the hemolymph was determined for 5 crayfish per sample time. For each sampling, 100 µl of hemolymph was aspirated in a calibrated capillary pipet which was inserted between coxa and basis of the left cheliped. From each animal sampling was carried out in duplicate. The blood glucose concentration was determined by the Glucose-GOD-Perid Methode (Test Combination Boehringer, Mannheim). The data were statistically analysed by the Student-t-test (two sided; significance accepted when $P<0.05$).

RESULTS

Hemolymph glucose level

During a 24 h period the hemolymph glucose level clearly shows a daily rhythmicity (Fig 1). During the dark period the glucose level reaches higher values than during the light period. It increases from around 22.00 h to reach a statistically significant maximum around midnight. In the remaining of the dark period a high blood glucose level is maintained until 6.00 h. During the period of light there are indications for a second rise, around 10.00 h, but this second maximum is not statistically significant.

Secretory activity of the CHH cells

Variations in the number of +, ++ and +++ cells are summarized in Fig 2. During a 24 h period the total number of immunopositive cells per eyestalk appears rather constant and fluctuates around 38 cells. About half of the immunoreactive cells can be characterized as ++ cells. For each sample the number of these ++ cells amounts to about 19. In contrast, the numbers of + and +++ cells per eyestalk vary around 8 and 10 cells, respectively, except at 18.00 h, 2 h before the beginning of the dark period. At that time they change drastically: the number of + cells increases significantly to around 18 cells, while the number of +++ cells drops to around 5 cells.

The mean cellular and nuclear volumes of the +, ++ and +++ cells during the 24 h cycle are presented in Figs 3 and 4. The ++ cells manifest the largest cell volume throughout the day/night cycle. In general, the +++ cells have the smallest cell volume and the + cells are intermediate in size. However, 2 h before the beginning of the dark period, the volume of the + cells increases significantly to a level comparable to that of the ++ cells. At 22.00 h the volumes of the three cell types have dropped, while after midnight their volumes increase again.

The mean nuclear volume of the +++ cells does not vary significantly during the whole day/night period and remains always the smallest. On the contrary, the mean nuclear volumes of the + and ++ cells show a sudden and significant decrease at 22.00 h.

Packing of the CHH axon terminals

The number of CHH granules present in the axon terminals is rather constant throughout the day/night period. However, at 23.00 h the packing of the CHH granules decreases significantly (Fig 5). Already after 1 hour the number of granules has been replenished to a level comparable to that found at the other investigated sample times.

DISCUSSION

The glucose level in the hemolymph of the crayfish, ***Astacus leptodactylus***, in intermoult stage, clearly shows a 24 h rhythmicity. Around midnight, the blood glucose concentration reaches a peak and during the remaining dark period the glucose level remains higher than during the light period. Two hours after the beginning of the light period we observed a consistent although smaller and not statistically significant peak. This rhythmicity is similar as has been described for ***Astacus leptodactylus*** by Strolenberg (1979). Comparable results were previously obtained for the crayfish ***Orconectes limosus*** (Hamann 1974) and the fresh water field crab ***Oziotelphusa senex senex*** (Reddy et al. 1981). For the latter two species it was further observed that the two blood glucose maxima coincide with peaks of locomotor activity. For ***Astacus leptodactylus*** we found the same relationship (unpublished results).

The remarkable differences in immunostaining intensity of CHH cells have previously been interpreted as a possible reflection of differences in the amount of antigen in individual CHH cells (Chapter I). In Chapter II we described variations in intensity of immunostaining among individual CHH cells within one animal for several other astacidean species and a palinuran species. Comparable results were recently obtained for CHH-producing neuroendocrine cells in a number of caridean species (Van Herp et al. in press). In a previous study described in Chapter III we could correlate these differences in immunoreactivity with varying amounts of neurosecretory granules in the cytoplasm and different secretory activity levels of the CHH cells. Poorly stained cells (+ cells) contain few CHH granules which are small but highly electron dense. They possess a large nucleus, which suggests the start of a period of synthetic activity. In the intermediate stage (++ cells) number and diameter of the CHH granules has increased. Such cells represent the stage where cells are most active in producing neurosecretory material. These cells have the largest volume. The cells showing the strongest immunoreactivity (+++ cells) have the highest content of CHH granules, while the electron density of these granules is lowest. The small volume of these cells indicates that biosynthetic activity is at the lowest level.

The interpretation of the cytological changes in the CHH-producing system in the eyestalk of ***Astacus leptodactylus*** observed in the present study is as follows:

1. The fact that about half of the total number of immunopositive cells is typified as ++ cells for all sample times suggests a continuous CHH production during the entire 24 h period. Such constant production of CHH seems very likely, as we may presume that CHH represents a neurohormone that intervenes in the physiological process on a daily basis.
2. The firm increase in the number of + cells and the decreased number of +++ cells 2 h before the onset of the dark period indicate that between 16.00 and 18.00 h a considerable amount of CHH material from the perikarya is transported into the tract.

3. Considering the mean cell volume of the three CHH cell stages during the 24 h period, we see at 18.00 h a firm increase of the mean cell volume of the + cells up to the level of the volume of the ++ cells. So, 2 h before the onset of the night, not only the number of + cells increases but also their volume. This indicates that these cells, after having transported their products into the tract, are stimulated to resume CHH synthesis. Another possibility may be that the above-mentioned cells, although immunocytochemically detectable as + cells, are in fact ++ cells that are synthetically active and transferring neurosecretory material at such a high rate into the axons that their immunoreactivity remains weak. However, both possibilities point to a supplementary stimulation of CHH synthesis around 18.00 h. Jaros (1977, 1980b) came to a similar conclusion in his autoradiographic study of the level of protein synthesis of the type A cells (later identified as CHH cells) of *Orconectes limosus* during a 24 h cycle with similar 12 h light and 12 h dark conditions. As in our experiments, he found the highest level of protein synthesis before the beginning of the dark period.

4. A previous electron microscopic investigation by Strolenberg (1979) on the amount of exocytotic activity of the type V granules (= CHH granules) containing axon terminals in the sinus gland of *Astacus leptodactylus*, revealed a burst of exocytoses around 20.00 h, the start of the dark period. This peak follows the above-mentioned proposed transport of CHH material from the perikarya into the axons two hours earlier, which would implicate that the granules need approximately two hours to migrate from the perikarya into the sinus gland. Similarly, Jaros (1977, 1980a) concluded that it takes about two hours for neurosecretory granules of *Orconectes limosus* to pass from the A cells (= CHH cells) in the MTGX-organ to the sinus gland. Considering the results on the numerical density of the CHH granules in the axon terminals, we see a firm decline of the packing at 23.00 h. If we combine these observations with the data on the exocytotic activity, we may assume that the increased release of CHH at the onset of the dark period, results in rather "empty" axon terminals three hours later. At the next sample time, at 24.00 h, the amount of CHH granules in the axon terminals appears to be replenished.

5. The burst of exocytoses at the onset of the dark period in *Astacus leptodactylus* implies a firm increase of CHH released into the hemolymph. This peak is followed, four hours later, by a peak in the hemolymph glucose level. This time interval is in agreement with previous results obtained by Strolenberg and Van Herp (1977), Strolenberg (1979) and Van Herp and Strolenberg (1980). These authors injected *Astacus leptodactylus* with either serotonin or d-c-AMP which evoked an increase in the number of exocytoses of the granule type V (= CHH granules). Hyperglycemia developed 2-4 hours later. Furthermore, injection of sinus gland extract caused an increase of blood glucose two hours after injection.

6. At 4.00 h the average cell and nuclear volumes of the + and ++ cells decline. These observations point to a reduction of synthetic activity at that time of the 24 h cycle. Two hours later the renewed increase of nuclear and cellular volumes suggests a resumption of the synthetic activity.

7. Strolenberg (1979) observed a second burst of exocytoses of the type V granules at 7.00 h which is followed by a second small peak in the blood glucose level around 10.00 h. Here again we see a period of approximately three hours between release of CHH and rise of the hemolymph glucose concentration.

8. The present results indicate that the daily changes in the synthetic activity of the entire group of CHH cells are not synchronous. In one MTGX-organ we find cells representing all stages of secretory activity throughout the 24 h period. Similar observations were reported by Jaros (1977; 1980b) who described large individual differences in the level of RNA and protein

synthesis in the type A cells (= CHH cels) in the eyestalk of the crayfish *Orconectes limosus* during a day/night cycle.

9. Our results further show that the total number of immunopositive cells per eyestalk remains rather constant at all sample times. However, CHH cells can only be identified if they contain immunoreactive material. CHH cells that do not contain neurosecretory granules will escape detection by means of the applied staining technique. Yet, as we find a constant number of immunoreactive cells during the 24 h period, we presume that we detect the total number of CHH cells present in the eyestalk.

In Fig 6 we illustrate our hypothesis concerning the 24 h rhythmicity of the secretory dynamics of the CHH cell group in the crayfish. During the major part of the daily period, the secretory activity of the CHH cells is rather constant. However, two hours before the beginning of the dark period, the transfer of CHH granules from the perikarya into the axons has increased and the new synthesis of CHH receives an extra stimulation (Fig 6, phase 1). The transferred CHH granules need two hours to migrate from the perikaryon to the axon terminals, where they arrive at the beginning of the dark period (Fig 6, phase 2). At this time a burst of exocytoses will cause a firm release of CHH into the hemolymph (Fig 6, phase 3). This peak of CHH release is responsible for the peak in the blood glucose concentration four hours later, at midnight (Fig 6, phase 4). At that time the secretory activity of the CHH cells is minimal. In the early morning period the synthesis of CHH increases again. One hour before the start of the light period a second burst of exocytoses takes place (Fig 6, phase 5). This increased release of CHH into the hemolymph will cause a second, less intense, blood glucose peak three hours later (Fig 6, phase 6).

LEGENDS

Fig. 1: Variations in the glucose value of the hemolymph during a 24 h period. Means (±S.E.M.) of 5 animals.

Fig. 2: Variations in the number of CHH cells according to their cell stage during a 24 h period. Means (±S.E.M.) of 5 animals.
——— = + cells; – – – – = ++ cells;
–·–·– = +++ cells; ····· = total number of cells.

Fig. 3: Variations in the cell volumes of the CHH cell stages during a 24 h period. Means (±S.E.M.) of 3 animals (5 cells per animal).
——— = + cells; – – – – = ++ cells;
–·–·– = +++ cells; ····· = total number of cells.

Fig. 4: Variations in the nuclear volumes of the CHH cell stages during a 24 h period. Means (±S.E.M.) of 3 animals (5 cells per animal).
——— = + cells; – – – – = ++ cells;
–·–·– = +++ cells; ····· = total number of cells.

Fig. 5: Variations in the packing of the granules in the CHH axon terminals during a 24 h period. Means (±S.E.M.) per μm^2 axon terminal measured for 5 animals. (Determined from photographs gently supplied by Dr. Strolenberg).

Fig. 6: Diagram illustrating the hypothetical 24 h rhythmicity of the CHH-producing neurosecretory system and the glucose concentration in the hemolymph, of the crayfish ***Astacus leptodactylus***.

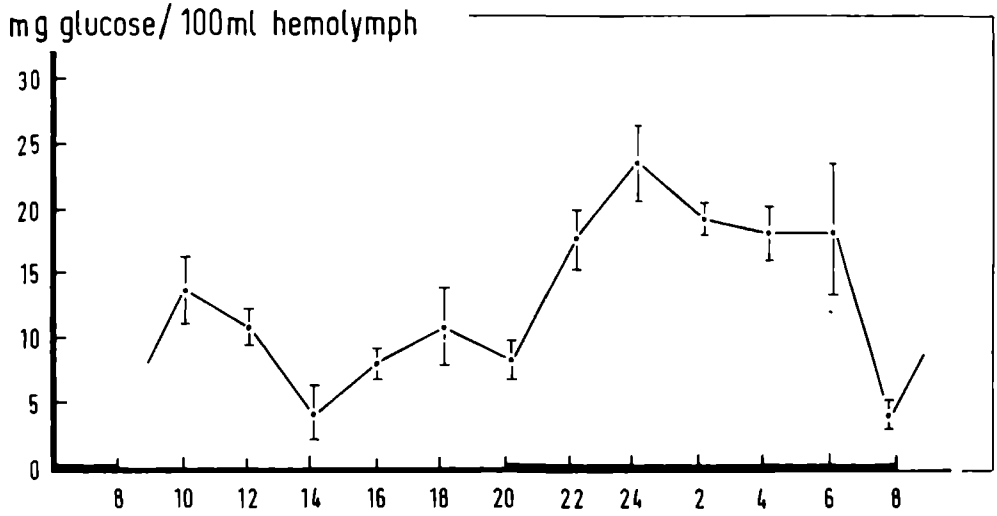

mg glucose/ 100ml hemolymph
30
25
20
15
10
5
0
8
10
12
14
16
18
20
22
24
2
4
6
8
t (hours)

Fig. 1

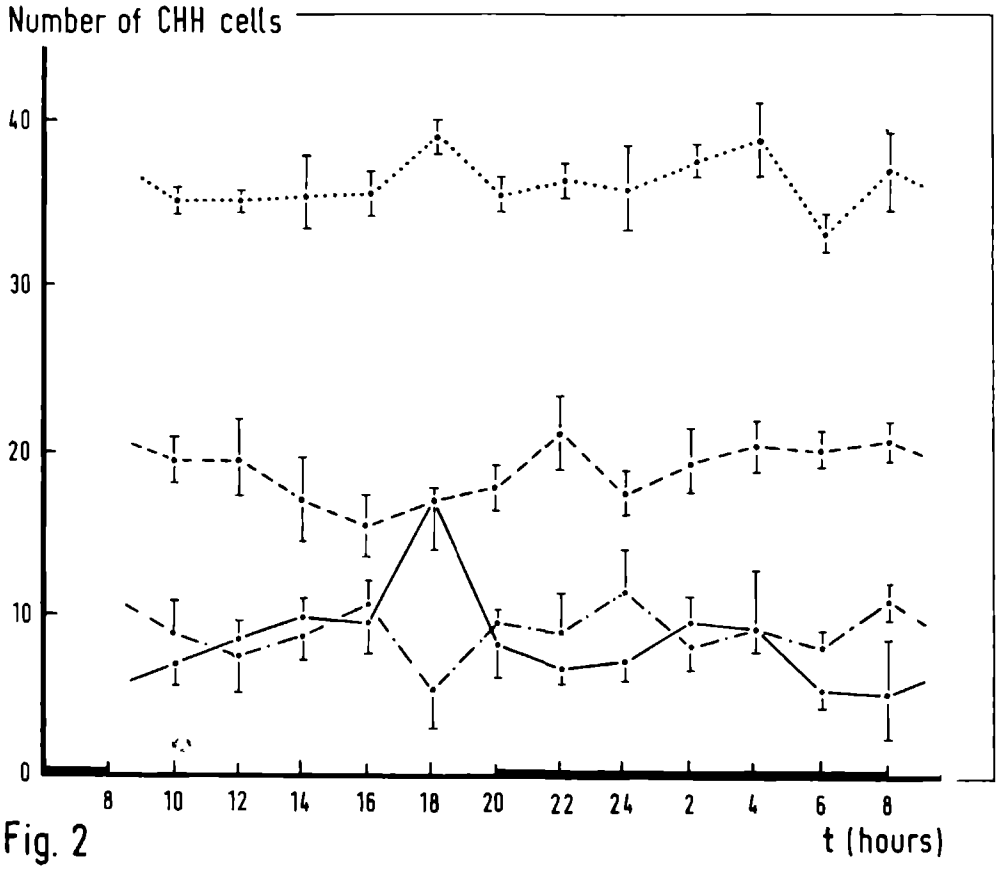

Number of CHH cells
40
30
20
10
0
8
10
12
14
16
18
20
22
24
2
4
6
8
t (hours)

Fig. 2

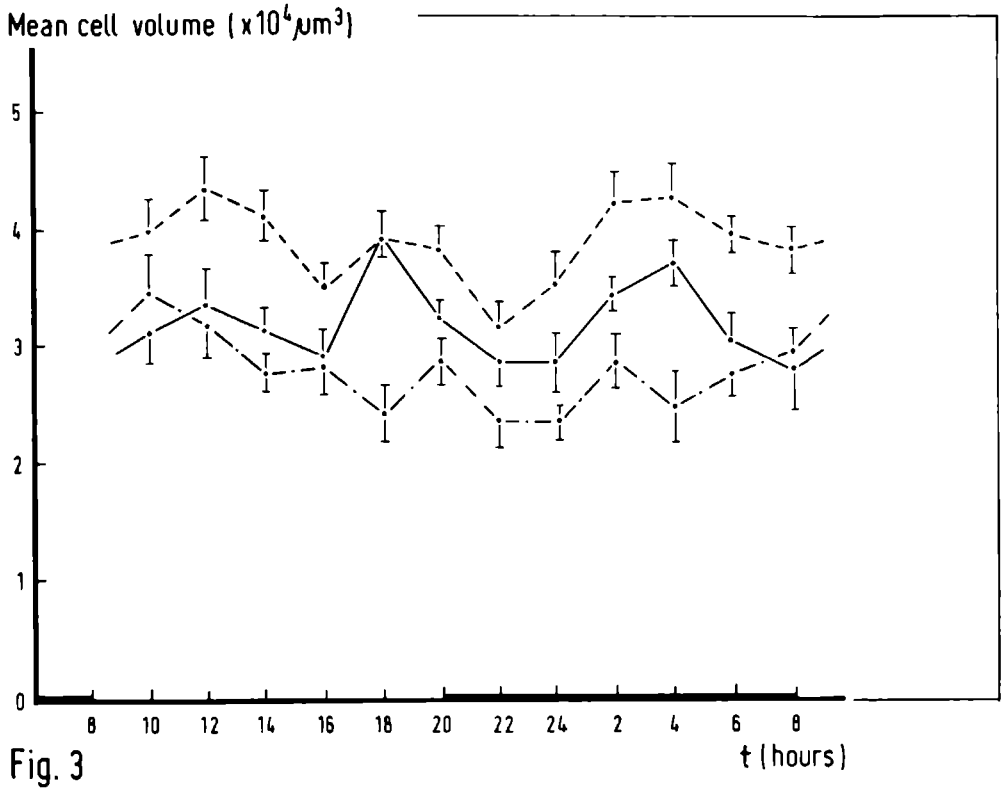

Mean cell volume (x10⁴µm³)
5
4
3
2
1
0
8
10
12
14
16
18
20
22
24
2
4
6
8
t (hours)

Fig. 3

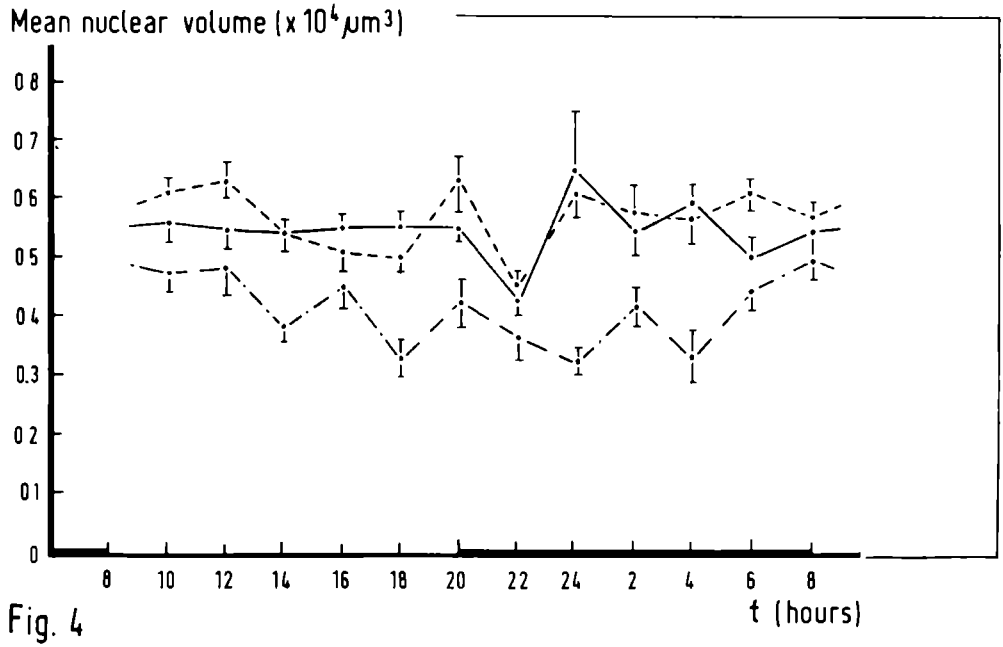

Mean nuclear volume (x 10⁴µm³)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
8
10
12
14
16
18
20
22
24
2
4
6
8
t (hours)

Fig. 4

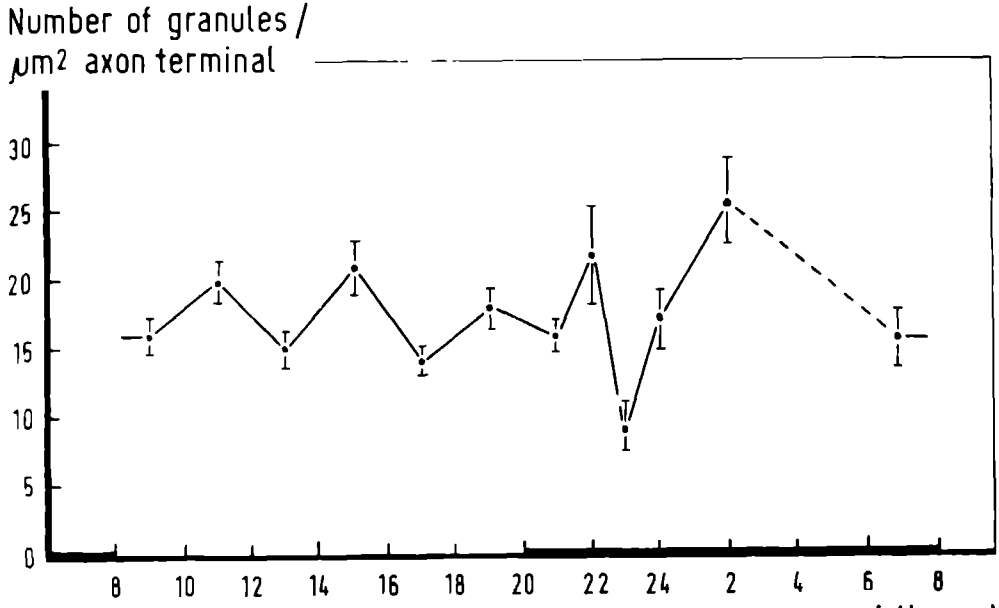

Number of granules /
µm2 axon terminal
30
25
20
15
10
5
0
8
10
12
14
16
18
20
22
24
2
4
6
8
t (hours)

Fig. 5

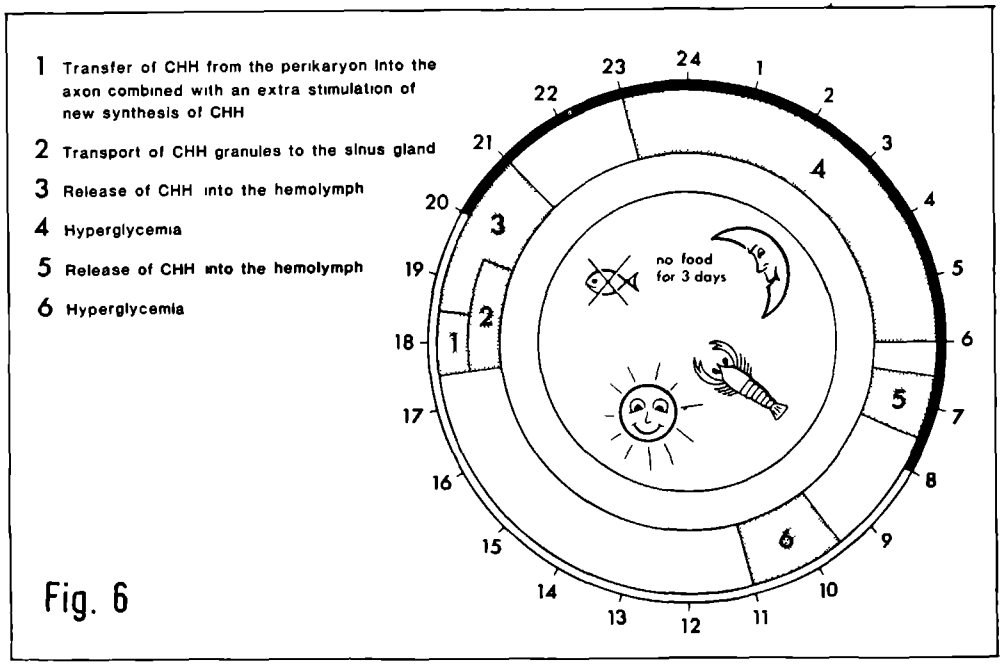

1 Transfer of CHH from the perikaryon into the axon combined with an extra stimulation of new synthesis of CHH
2 Transport of CHH granules to the sinus gland
3 Release of CHH into the hemolymph
4 Hyperglycemia
5 Release of CHH into the hemolymph
6 Hyperglycemia
no food
for 3 days

Fig. 6

CHAPTER V

SECRETORY ACTIVITY OF THE CRUSTACEAN HYPERGLYCEMIC HORMONE-PRODUCING SYSTEM IN THE EYESTALK OF THE CRAYFISH ASTACUS LEPTODACTYLUS, DURING THE MOULTING PERIOD

SUMMARY

During an autumn moulting period the secretory activity of the CHH-producing system in the eyestalk of the crayfish *Astacus leptodactylus*, and the fluctuations in the blood glucose level were studied for each stage of the moulting period.
Furthermore, the blood glucose level for each moulting stage was examined during a 24 h period.

The nocturnal hyperglycemia is completely absent in every moulting stage. The activity of the CHH cells is not correlated with any of the moult stages. In each moulting stage the nuclear volumes of all CHH cells are strongly decreased.

The disappearance of the nocturnal hyperglycemic peak may be due to a modulating effect of ecdysteroids on the blood glucose level, since in preliminary experiments it was shown that β-ecdyson present in the ambient water evokes the disappearance of nocturnal hyperglycemia.

INTRODUCTION

In the life of decapod crustaceans moulting represents an intervening process which is more than just a brief interruption of the normal life pattern. Studies on the physiology of decapods revealed a multitude of functions such as reproduction, osmoregulation, enzymatic activities, Ca-metabolism, protein, lipid and glycogen metabolism, that are influenced by, or undergo cyclic changes related to the moulting process (for reviews see Passano 1960; Keller 1974; Kleinholz and Keller 1979).

With respect to the synthesis of the exoskeleton, special attention has been paid to the carbohydrate metabolism during moulting. Previous studies showed the important role of blood glucose and glycogen supplies for the chitin synthesis of the new cuticle (Scheer and Scheer 1951; Travis 1957; Hu 1958; Parvathy 1971a). Further investigations revealed that N-acetyl-glucosamine, liberated by hydrolysis of the old exoskeleton, functions as the main source for chitin synthesis during premoult. During this premoult period, N-acetyl-glucosamine also serves as the predominant energy source. Conversely, during postmoult, glucose serves as the main precursor for chitin synthesis (Lang 1971; Stevenson and Tung 1971; Speck et al. 1972; Gwinn and Stevenson 1973; Herz-Hübner and Ürich 1973). Determination of the hemolymph glucose level during the moulting cycle revealed a marked rise in late premoult (Drilhon 1933, 1935; Renaud 1949; McWhinnie and Saller 1960; Robertson 1960; Andrews 1967; Telford 1968a,b, 1974; Parvathy 1970, 1971b; Van Herp et al. in press).

During the intermoult period, the crustacean hyperglycemic hormone (CHH) is involved in the regulation of carbohydrate metabolism (Sedlmeier and Keller 1981; Sedlmeier 1982). In Chapter IV, we demonstrated a daily rhythmicity in the blood glucose level and in the secretory activity of the CHH-producing system during the intermoult period for the crayfish *Astacus leptodactylus*. However, the significance of the CHH during the moulting process is unknown.

In this chapter we present some observations on the secretory activity of the CHH-producing cells in relation to the fluctuations in the blood glucose level during the moulting period. In addition, the 24 h rhythmicity of the hemolymph glucose was determined for each moulting stage.
In an addendum, preliminary observations are reported that indicate that ecdysteroids have a modulating effect on the daily blood glucose rhythmicity.

Animals and blood sampling

Crayfish of the species ***Astacus leptodactylus*** were obtained commercially and kept in the laboratory as described in Chapter I. Experiments were performed with adult male and female crayfish of equal size, adapted to 12 h light and 12 h dark conditions. Adult ***Astacus leptodactylus*** moult once or twice a year. The predominant moult for both males and females takes place in October and November. The animals can moult a second time generally in the period February to April. Spring moult of females which carry fertilized eggs is delayed and occurs in the period April and May, not before the juvenils have left the pleiopods of the breeding female. Occasionally, animals are found which moult during the summer. Determination of the different moult stages occured after the method of Drach (1944) as adjusted for ***Astacus leptodactylus*** by Van Herp and Bellon-Humbert (1978) and is based upon the morphological changes in setal development. With these morphological criteria, the moulting cycle was divided in A, B (postmoult stages), C (intermoult stage), D_0, D_1', D_1'', D_1''', D_2, D_3, D_4 (premoult stages) and E (ecdysis).

For animals in autumn moult the hemolymph glucose levels for each moulting stage were determined at 14.00 h as well as during a 24 h period (comparable with the experiment from Chapter IV). Crayfish were selected on the basis of their moulting stage. The total number of specimens available per moulting stage was variable and often limited. As a consequence the total number of sampling groups per moulting stage varied, which implicated that blood samples were taken at time intervals varying between 2 and 8 hours. Furthermore, the number of specimens per sample group varied. Details on the number of specimens per sample are given in the legends of the presented graphs (Results section). To have an idea about the 24 h rhythmicity during the spring moult, some samples times were also checked during the spring moult period. The blood glucose levels were determined using the Glucose Quant Test Combination (Boehringer Mannheim GmbH).

Light microscopy

In order to study the relation of the secretory activity of the CHH cells with the moulting stages, eyestalks were ablated at 14.00 h of animals in each stage of the autumn moult. Fixation, dehydration, embedding in paraplast and the immunocytochemical staining procedure for the 7 µm sections were performed according to the methods described in Chapter I. To determine the optimum dilution of the primary antiserum for the different moulting stages, the primary antiserum was applied in a dilution series (1/50, 1/150, 1/500 and 1/1000), which revealed an optimum dilution of 1/150 for all moulting stages.

The secretory cell stages of the CHH cells were determined on the basis of the observed differences in staining intensity as described in Chapter III. For each moulting stage the number of +, ++ and +++ cells was counted for one eyestalk of 3 animals. In addition the cellular and nuclear volumes of 5 +, 5 ++ and 5 +++ cells per eyestalk were determined according to the method described in Chapter III.

Blood glucose levels and secretory activity of the CHH cells

Data on the hemolymph glucose concentration, the number of cells of each cell stage (+, ++, +++) and the cellular and nuclear volumes determined at 14.00 h for each stage of the autumn moulting period are presented in Fig1 A-D. The data are compared with those from the autumn intermoult animals obtained at 14.00 h, as presented in Chapter IV.

For all moulting stages the glucose concentrations are comparable, except in the premoult stages D_2-D_4 when a firm elevation is observed with a maximum in stage D_3 (Fig 1A). In each moulting stage, the total number of immunopositive cells appears rather constant and varies around 38 cells. The number of + cells is increased during the premoult period and decreases firmly in stage A. The number of ++ cells is slightly decreased during the premoult period. The number of +++ cells is slightly increased during the total moulting period (Fig 1B). We observed that the cell volumes of the + and ++ cells decrease during late postmoult and early premoult. The mean nuclear volumes of all CHH cells are decreased during the total moulting period with a minimum during premoult where we observed nuclear volumes decreased with 30-50%. The standard error of the morphometric data presented in Fig1B-D often shows considerable differences.

The morphometric data obtained for the animals in spring moult, although limited, are in line with the above presented results for the autumn period.

The intensity of the immunostaining of the tract and the sinus gland did not fluctuate in any moult stage and is comparable to that observed in summer intermoult animals.

Blood glucose rhythmicity during a 24 h period.

For all investigated moulting stages the glucose levels are similar; a nocturnal peak is absent (Figs 2-8). The observed rise in the blood glucose concentration in stage D_3 at 14.00 h presented in Fig 1A, is consistent for the total 24 h period. The mean blood glucose concentrations per sample time are considerably more variable during the premoult stages D_2-D_4 than during the other moulting stages.

The preliminary data on the 24 h rhythmicity of the blood glucose level for animals in spring moult are similar to those in autumn animals. For both autumn and spring moult no differences were found between the blood glucose levels of males and females.

DISCUSSION

The present results on the glucose levels observed at 14.00 h for each stage of the moulting cycle show a blood glucose level that is similar for each moulting stage and comparable with the glucose concentration determined for the summer intermoult animals (Chapter IV). Only in the premoult stages D_2-D_4 we found a firm rise. This latter glucose elevation has been described previously for several decapods (Drilhon 1933, 1935; Renaud 1949; McWhinnie and Saller 1960; Robertson 1960; Andrews 1967; Telford 1968a,b, 1974; Parvathy 1970, 1971b).

Our observations on the secretory activity of the CHH cells of animals fixed at 14.00 h reveal a slightly increased number of +++ cells and considerably reduced nuclear volumes of all CHH cells in all stages of the moulting period if compared to the data found for the summer intermoult. These data point to an increased storage of CHH in the perikarya and a reduced synthetic activity of all CHH cells during the whole moulting period. However, we did not observe changes in synthetic activity of the cells that might account for the observed glucose peak in D_3. This may be caused by the particular point of time selected for sampling (14.00 h).To obtain more information on the activity of the CHH cells and the release of CHH, we extended our study with observations on the 24h rhythmicity of the hemolymph glucose level, as this is a parameter for the activity of the CHH cells (Chapter IV). These experiments revealed that, for both the animals in autumn and spring moult, the nocturnal peak is completely absent. These results, in combination with the data on the secretory activity of the cells, lead to the supposition that the functioning of the CHH cells and the release of CHH into the hemolymph as in summer intermoult animals (Chapter IV) is different during the moulting period. This assumption is not in line with the results on the CHH-producing cells of the prawn ***Palaemon serratus*** (Van Herp et al. in press). These authors describe an inverse relationship between the number of immunoreactive cells and the blood glucose level during different stages of the moulting cycle.

Changes in CHH functioning and carbohydrate metabolism during moulting have previously been indicated. Scheer (1959) assumed from experiments on the crab ***Carcinides maenas (Carcinus maenas)*** that the content of the eyestalk factor involved in carbohydrate regulation is lower during premoult stages than in the intermoult stages. According to Keller and Beyer (1968) and Keller (1974) the increase in blood glucose of the crayfish ***Orconectes limosus*** observed after injection of serotonin or eyestalk extracts during the intermoult does not occur during the moult. Therefore, Keller (1974) assumed that the sensitivity of the target tissues for CHH must be different during moulting. Similar observations were made by Parvathy (1972). His experiments with the crab ***Ocypoda platytarsis*** revealed that eyestalk ablation in intermoult caused a strong decrease in blood glucose content, while the same experiment performed in premoult had no effect on the glucose concentration. Results of Strolenberg (1979) indicated a decrease of the synthesis and release of neurosecretory hormones during the moulting period for ***Astacus leptodactylus***. In this respect it is of interest to mention the results of Speck and Urich (1971, 1972) who determined for the crayfish ***Orconectes limosus*** that N-acetyl-glucosamine, liberated by resorption of the old exoskeleton and occuring in the hemolymph in large amounts during premoult, functions as the only source of energy for metabolism during the main period of the moulting cycle. This finding can be an explanation for the proposed inactivity of the CHH system: its functioning would be superfluous during the moulting period. Furthermore, the resorption of the old cuticle, which is maximal during late premoult, might be the source of the glucose peak in stage D_3.

According to the classic scheme of moult control in crustaceans, during intermoult ecdyson titers are depressed by a moult inhibiting hormone (MIH) produced by neurosecretory cells in the eyestalk. Moulting is induced by a decreased release of MIH which results in the production of more α-ecdyson in the Y-organ. This product is rapidly hydroxylated in several tissues to β-ecdyson (20-hydroxyecdyson or crustecdyson) which is the principal biologically active form of the hormone (for a review see Kleinholz and Keller

1979). McCarthy and Skinner (1979) studied the metabolism of α-ecdyson in intermoult land crabs *(Gecarcinus lateralis)*, and determined that free and conjugated ecdysteroids (e.g. β-ecdyson) are excreted in both the urine and feces. McCarthy (1982) concluded for the same above-mentioned species that the rate of elimination of serum ecdysteroids into the ambient water is maximal at about 24 to 48 h before ecdysis. Kittredge and Takahashi (1972) presented indications that β-ecdyson might function as a pheromone. They found that the presence of this steroid in the water influences the sexual behaviour of male decapods in concentrations up to 10^{-13} M (for a review see Willig 1974). Furthermore, the assumption has been made previously that β-ecdyson influences the activity of the hyperglycemic hormone (Parvathy 1972).
With this in mind, we postulate that the moulting hormone affects blood glucose levels. In this respect we started a pilot study to test the possible modulating effect of ecdysteroids on the daily blood glucose rhythmicity. These experiments are described in the following addendum.

THE EFFECT OF ECDYSTEROIDS ON THE BLOOD GLUCOSE RHYTHMICITY

To obtain information on the possible effect of ecdysteroids, excreted in the water by moulting animals, on the regulation of the daily blood glucose rhythmicity, we kept a group of male crayfish in summer intermoult (experiment performed in July) for one week in a separate tank filled with tap water to which we added β-ecdyson (Rohto Pharmaceutical Co, Ltd, Rosaka, Japan) in a concentration of 5×10^{-8} M. A second group of control animals was kept in another separate tank in tap water. Both groups were kept under 12 h light and 12 h dark conditions. Blood samples were taken for both experimental and control animals during a 24 h period at time intervals of 4 h, for 5 crayfish per sample point.

The results are presented in Fig 9. For the control experiment a 24 h blood glucose rhythm is observed with a peak at midnight. However, the glucose level of the animals kept in contact with β-ecdyson does not demonstrate this usual 24 h rhythm.

In addition, we extended these preliminary observations with an experiment to test possible pheromone functioning of products excreted by a population of moulting crayfish. For that purpose we kept crayfish in intermoult stage for a period of one month in contact with moulting animals. After this period, blood samples were taken from the intermoult animals during a day/night cycle at intervals of 4 h, for 5 crayfish per sample time. The results show the absence of the nocturnal peak that is typical for controls (Fig 10).

These results indicate that moulting hormone affects carbohydrate metabolism. Moreover, moulting hormone excreted by moulting animals may act as a pheromone that changes carbohydrate metabolism of surrounding animals. Further investigation of this interesting possibility is required. Questions that need to be answered pertain the biochemical nature and the mechanism of action of the ecdysteroids in influencing carbohydrate metabolism and the CHH system. Moreover, the production rate of these substances during the various moulting stages has to be established. Such studies are in progress in our laboratory.

Fig. 1A-D: The secretory activity of the CHH cells at 14.00 h compared with the variations in the blood glucose level at 14.00 h, determined during the autumn moulting cycle for the crayfish *Astacus leptodactylus*. The data are compared to the results obtained for the summer intermoult animals described in chapter IV (C*). Fig 1A: Variations in the glucose values in the hemolymph during the moulting period. Means (±S.E.M. of 5 animals per moulting stage). Fig 1B: Variations in the number of CHH cells according to their cell stage during the moulting period. Means (±S.E.M.) of 5 animals (one eyestalk per animal). Fig 1C: Variations in the cell volumes of the CHH cell stages during the moulting period. Means (±S.E.M.) of 3 animals (one eyestalk per animal, 5 cells per animal). Fig 1D: Variations in the nuclear volumes of the CHH cell stages during the moulting period. Means (±S.E.M.) of 3 animals (one eyestalk per animal, 5 cells per animal).

Figs 2-8: Hemolymph glucose levels during a 24 h period for crayfish in different autumn moulting stages, kept under constant 12 h light/ 12 h dark conditions. Means (±S.E.M.). Fig 2: D_0 (n=4 at t=10, 16, 20, 4 h n=5 at t=12, 24, 8 h). Fig 3: D_1'-D_1''' (n=4 for all sample times). Fig 4: D_2 (n=3 for all sample times). Fig 5: D_3 (n=4 at t=12, 16, 24, 4 h; n=5 at t=10, 20, 8 h). Fig 6: D_4 (n=3 at t=10, 12 h n=5 at t=16, 20, 24, 4, 8 h). Fig 7: A (n=5 for all sample times). Fig 8: B (n=8 at t=10, 12, 24, 4 h; n=9 at t=16, 20, 8).

Fig. 9: Comparison of the daily hemolymph glucose rhythm of crayfish in summer intermoult stage kept under control conditions and in the presence of β-ecdyson (means ±S.E.M. n=5 for all sample times; experiment performed in August).

Fig. 10: Hemolymph glucose levels during a 24 h period for crayfish in intermoult stage, kept in contact with animals going through moult (means ±S.E.M.; n=5 for all sample times; experiment performed in November).

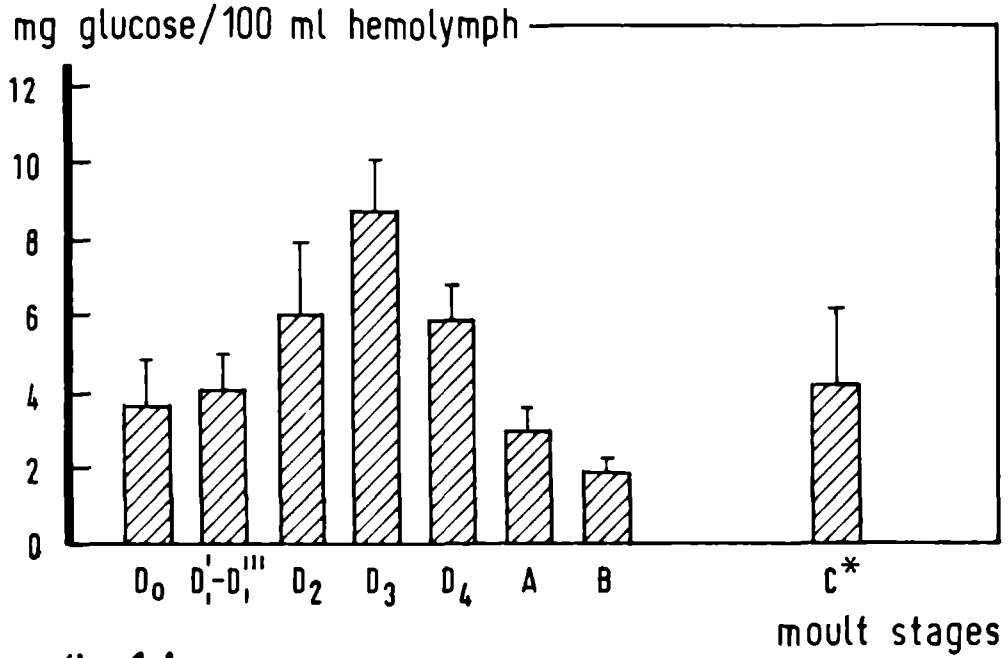

mg glucose/100 ml hemolymph
12
10
8
6
4
2
0
D_0
D_1'-D_1'''
D_2
D_3
D_4
A
B
C*
moult stages

fig. 1A

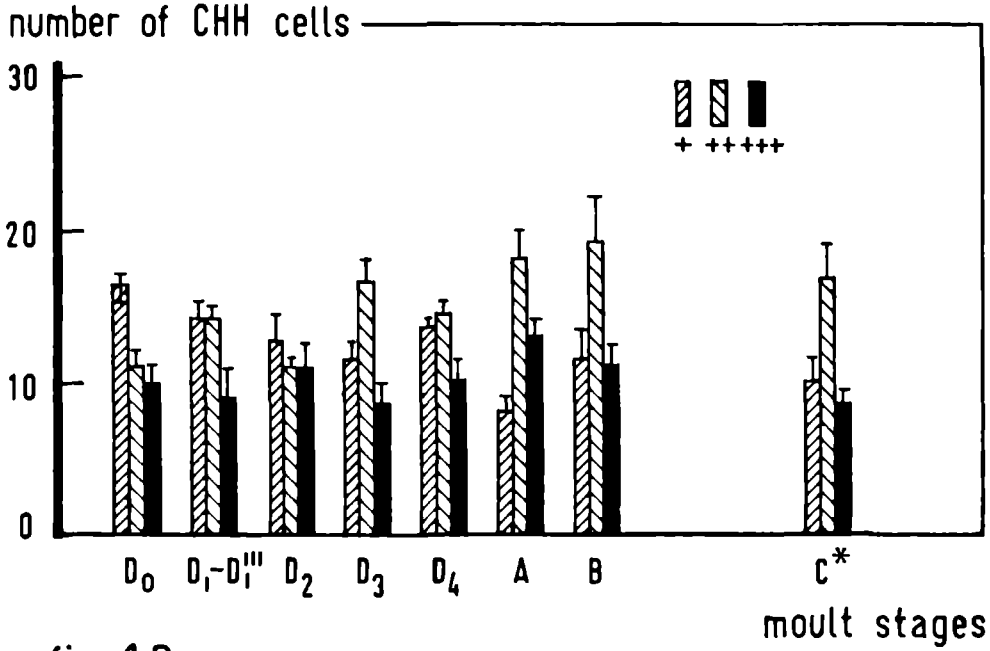

number of CHH cells
30
20
10
0
+ ++++
D_0
D_1-D_1'''
D_2
D_3
D_4
A
B
C*
moult stages

fig. 1B

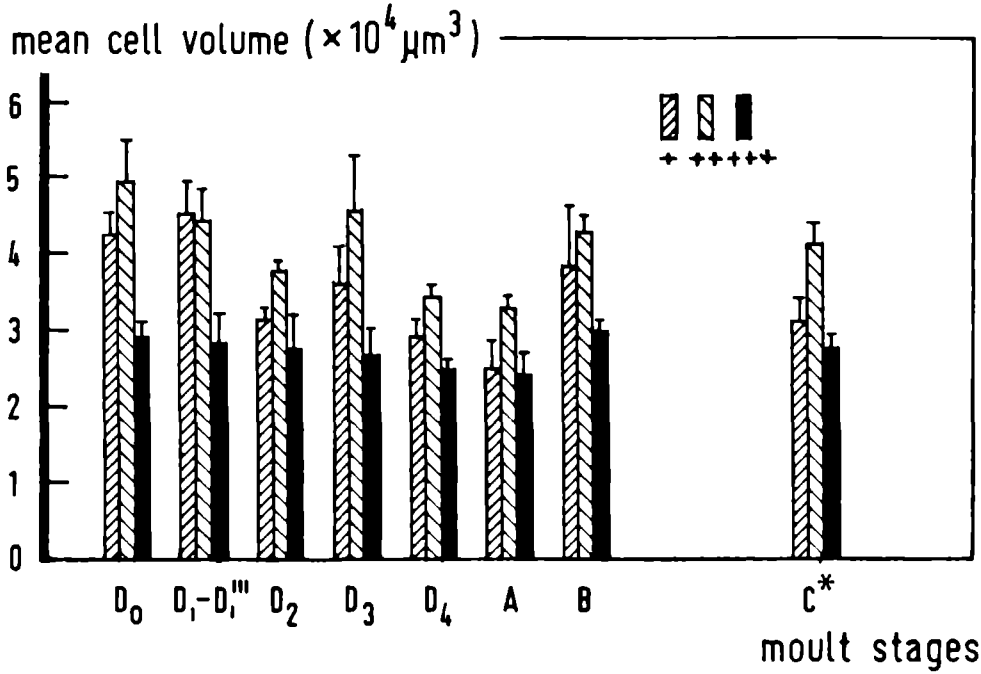

mean cell volume ($\times 10^4 \mu m^3$)
6
5
4
3
2
1
0
+ ++++
D_0 D_1-D_1''' D_2 D_3 D_4 A B C*
moult stages

fig. 1C

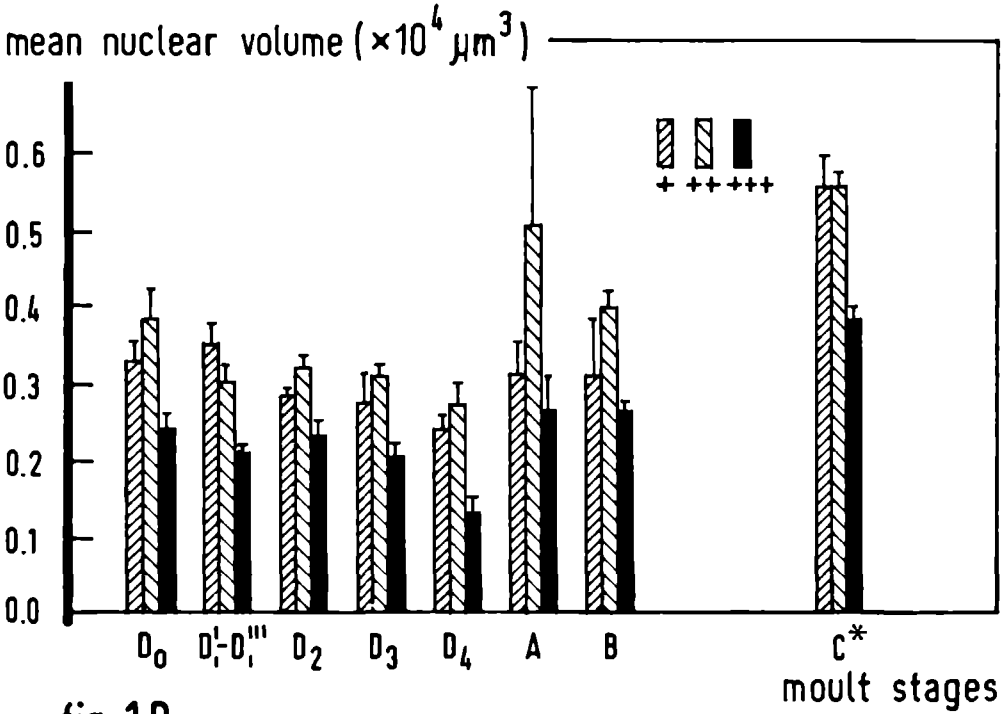

mean nuclear volume ($\times 10^4 \mu m^3$)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
+ ++ +++
D_0 D_1'-D_1''' D_2 D_3 D_4 A B C*
moult stages

fig. 1D

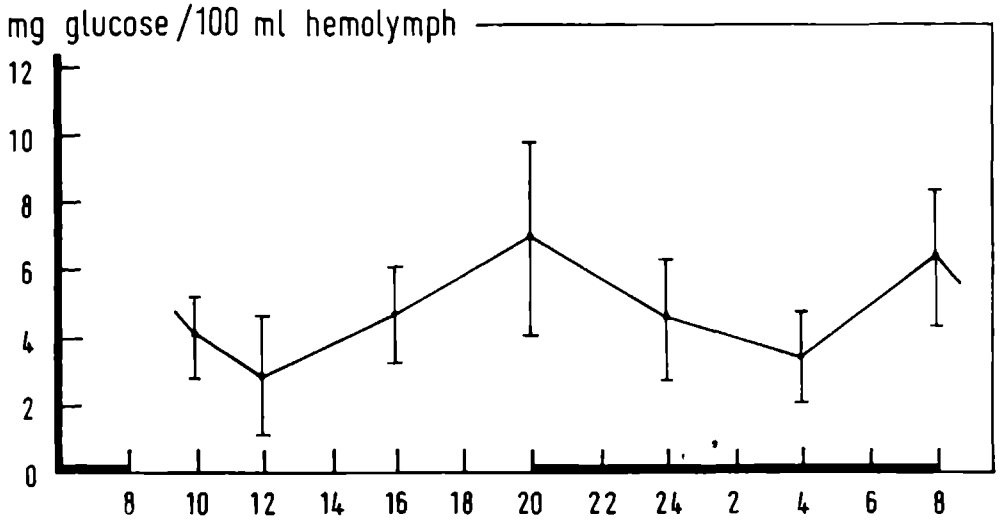


fig. 2 (D_0)

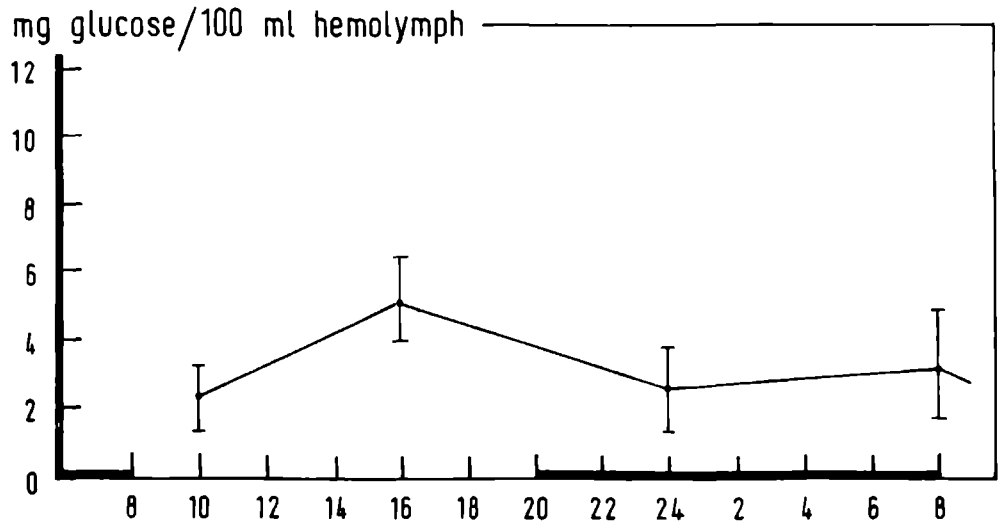


fig. 3 ($D_1^{'}-D_1^{'''}$)

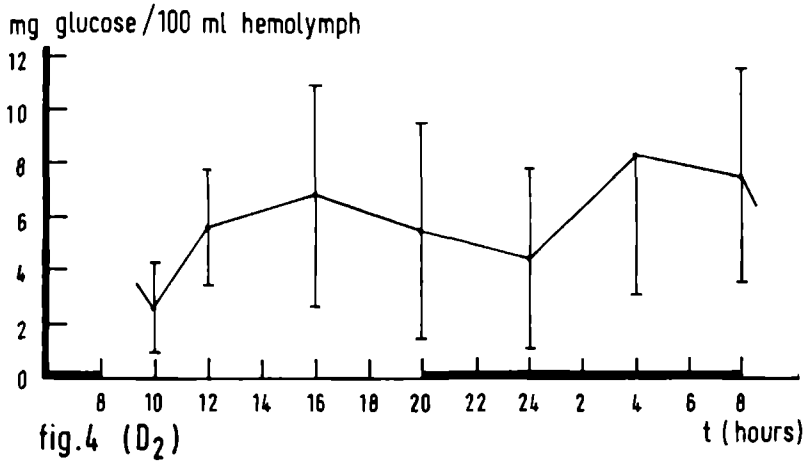


fig.4 (D_2)

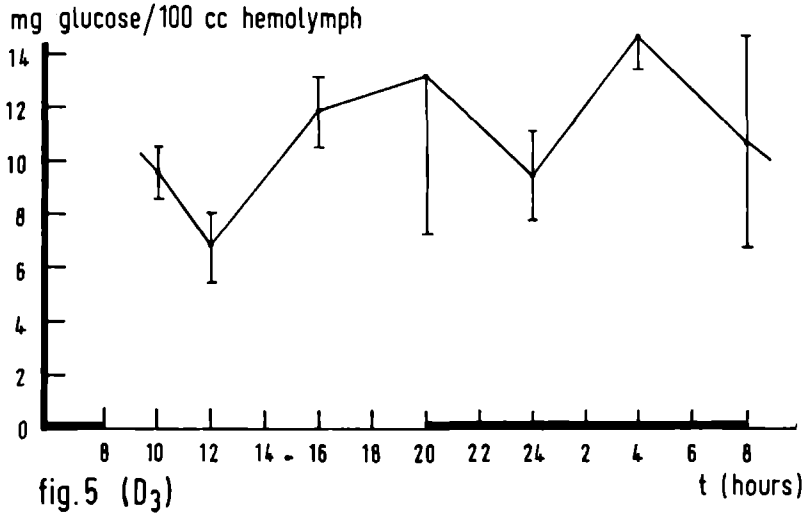


fig.5 (D_3)

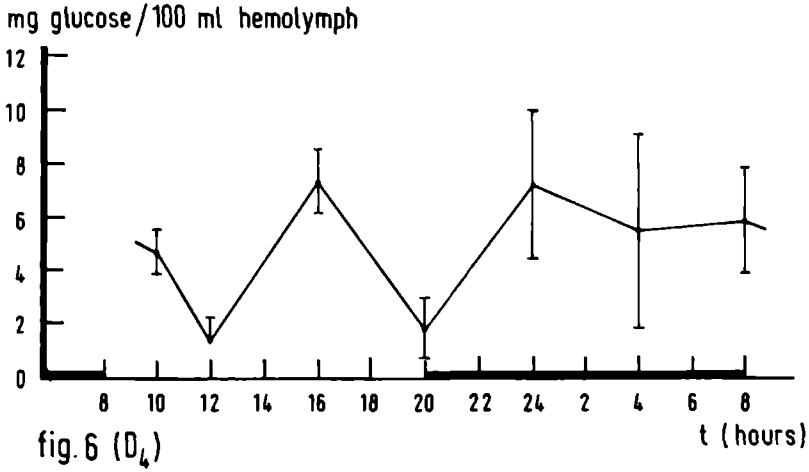


fig.6 (D_4)

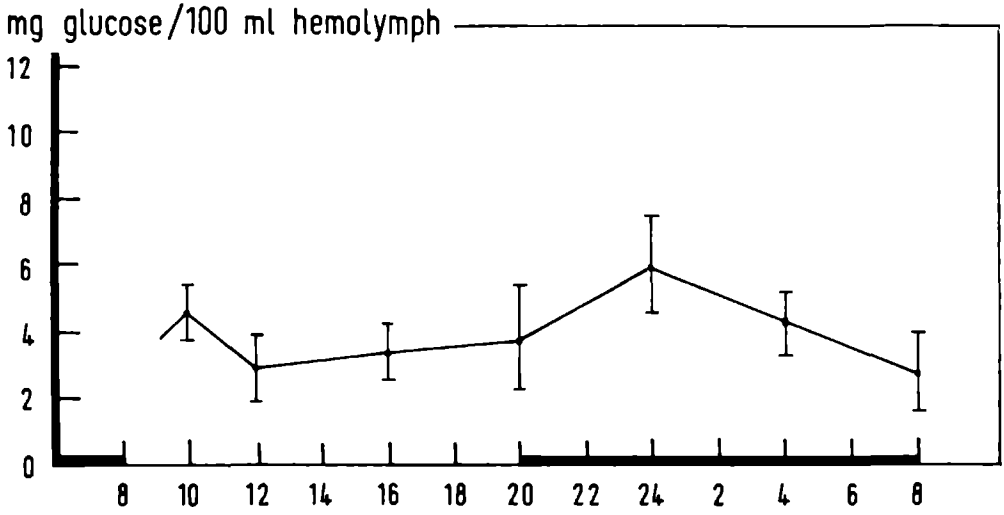
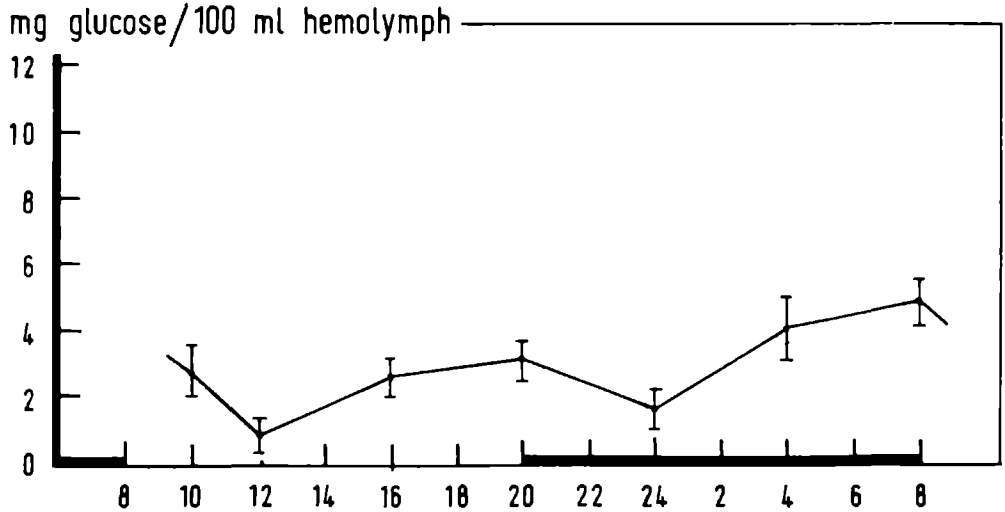

mg glucose/100 ml hemolymph
12
10
8
6
4
2
0
8
10
12
14
16
18
20
22
24
2
4
6
8
t (hours)

fig. 7 (A)


mg glucose/100 ml hemolymph
12
10
8
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2
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12
14
16
18
20
22
24
2
4
6
8
t (hours)

fig. 8 (B)

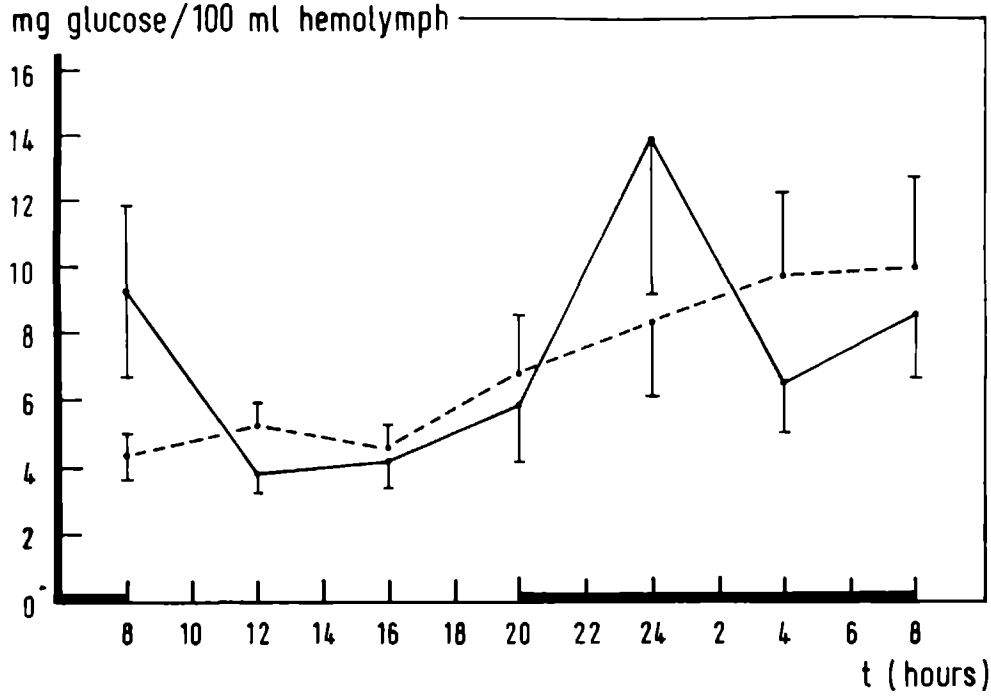


fig.9 (—control; ---β-ecdyson)

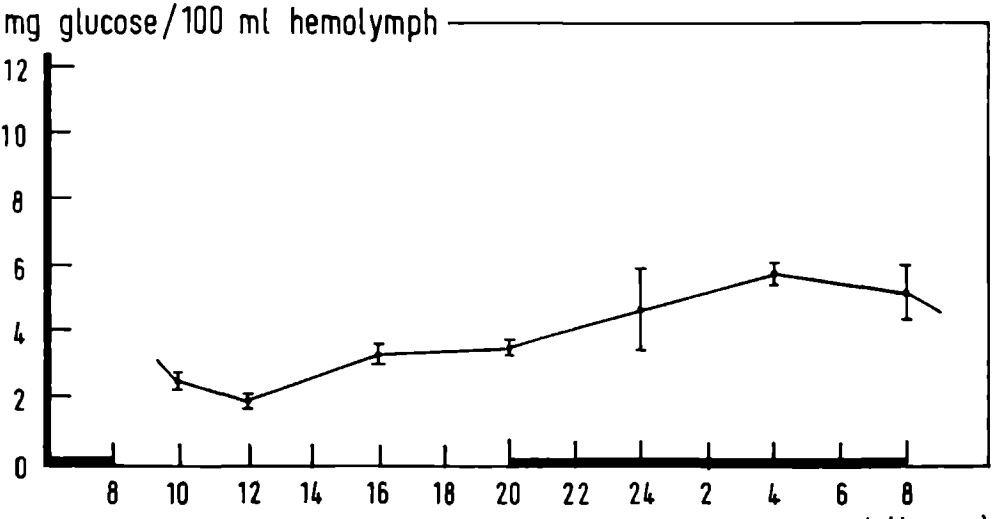


fig. 10 (C)

CHAPTER VI

THE CRUSTACEAN HYPERGLYCEMIC HORMONE-PRODUCING SYSTEM DURING LARVAL AND POSTLARVAL DEVELOPMENT IN THE EYESTALK OF THE CRAYFISH ASTACUS LEPTODACTYLUS, AS STUDIED BY IMMUNOCYTOCHEMISTRY

Gorgels-Kallen and Meij (1985),
Journal of Morphology 183, in press.
Presented at the meeting of the
Dutch British Endocrine Society, August 1982:
Gorgels-Kallen and Van Herp (1982).

SUMMARY

The eyestalks of the larval and postlarval stages of the crayfish ***Astacus leptodactylus*** were immunocytochemically investigated, with an anti-***Astacus***-CHH serum. Already in the larval stage, immediately after hatching, immunopositive staining occurs in neurosecretory cells of the medulla terminalis ganglionic X-organ (MTGX), in an MTGX-sinus gland tract and in part of the axon terminals in the sinus gland. During postlarval development the number of these immunopositive cells increases. Immunocytochemical investigation of the neurohemal region at the electron microscopic level revealed immunostaining in neurosecretory granules located in axon terminals. During postlarval development the mean diameter of the stainable granules increases. Injection of larval eyestalk extract in adult crayfish results in a rise of the hemolymph glucose concentration.

INTRODUCTION

There is an extensive literature on hormonal regulation by the X-organ sinus gland complex in adult decapod crustaceans. Contrastingly, knowledge of the developmental morphology, physiology and neurosecretory activity of the X-organ sinus gland complex during larval and postlarval development is limited.

At the light microscopic level, the neurosecretory system in larval eyestalks of a few decapod species has been described. Pyle (1943) observed the X-organ already in the egg and larval stages of a crab ***(Pinnotheres maculatus)*** and a lobster ***(Homarus americanus)***. He could detect the sinus gland as a well defined structure only up from the third stage after hatching in ***Homarus***, and only in the adult stage of ***Pinnotheres***. Contrastingly, Matsumoto (1958) observed not only the X-organ but also the sinus gland in the crab ***Potamon dehaani*** directly after hatching. Hubschman (1963) studied the larval stages of some prawn species ***(Palaemonetes)***. He could not detect the X-organ before the adult stages; the sinus gland was not recognizable before the fifth larval stage. Jacques (1969) could not detect the X-organ nor the sinus gland in any larval stage of the stomatopodes ***Lysiosquilla occulta***, ***Squilla mantis*** and ***Squilla desmaresti***. Zielhorst and Van Herp (1976) described the presence of the X-organ for the crayfish ***Astacus leptodactylus*** directly after hatching and they discovered the sinus gland as a well developed structure in the third postlarval stage. Bellon-Humbert et al. (1978) studied the ontogenesis of the eyestalk structures in the prawn ***Palaemon serratus***. They could not recognize the sinus gland before stage five and they did not observe the differentiation of the medulla externa X-organ (MEX) and MTGX before metamorphosis.

At the ultrastructural level, Jacques (1975) has described the sinus gland in the first larval stage of the stomatopode ***Squilla mantis***. Strolenberg (1979) studied the ultrastructure of the sinus gland of the crayfish ***Astacus leptodactylus*** during larval and postlarval development. He was able to detect the sinus gland immediately after hatching. He described the development of the different neurosecretory granule types and compared them with those in the adult crayfish.

Little is known about the presence and physiological activity of the neurosecretory hormones during the larval development of crustaceans. Several investigators studied physiological processes in larval decapods, that are known to be hormonally regulated in adults. They did not always find conclusive evidence as to whether or not these processes already are hormonally regulated in larvae. The studies of Broch (1960) on ***Palaemonetes vulgaris***,

Costlow (1961) on brachyuran larvae and Costlow and Sandeen (1961) on eggs of *Sesarma reticulatum* show that the regulation of the chromatophores is already affective in early stages of development. Costlow (1966) postulated the appearance of a moult inhibiting hormone from stage three in the mud crab *Rhithropanopeus harrisii*.

So far, the presence and biological activity of the crustacean hyperglycemic hormone (CHH) in larval eyestalks is unknown. In adult decapods hyperglycemic activity of eyestalk extract was already reported in 1944 by Abramowitz et al. Since that time biological activity and molecular structure of the CHH material have been intensively studied (Kleinholz and Keller 1979; Keller 1981; Sedlmeier and Keller 1981; Sedlmeier 1982; Newcomb 1983; Van Wormhoudt et al. 1984a,b; Chapter VIII). The past few years our knowledge on the location of the CHH-producing system in adult decapods has increased greatly (for *Astacus leptodactylus* see Van Herp and Van Buggenum 1979; Chapter I; for a brachyuran species see Jaros and Keller 1979; for some astacidean species and a palinuran species see Chapter II; for some caridean species see Van Herp et al. in press).

The aim of the present study is to extend our knowledge of the developmental morphology of the CHH neurosecretory system in the eyestalk of the larval and juvenile stages of the crayfish *Astacus leptodactylus*. By the aid of an anti-*Astacus*-CHH serum we studied the morphology of immunoreactive neurosecretory cells at the light microscopic level. Ultrastructurally, we described the presence of the immunopositive neurosecretory granules in the sinus gland. Furthermore, we tested the larval eyestalk extract on hyperglycemic activity.

MATERIALS AND METHODS

Animals

Crayfish of the species *Astacus leptodactylus* were obtained commercially and kept in the laboratory as described in Chapter I. Female crayfish with fertilized eggs were kept in separate tanks. After hatching the larvae remain inactive and do not eat as, in that period, they are supplied with an important vitellin store. In this developmental stage (called stage L: it is characterized by the occurrence of sessile eyes and a telson without uropods) the larvae remain attached to the female pleiopods until the first moult, which occurs about 10 days after hatching. At this stage (called stage I) the juveniles are able to leave the female pleopods, to swim and to eat. They have stalked eyes. By that time the young animals were collected and kept in separate tanks in running tap water. They were fed three times a week with nauplii of *Artemia*, with small pieces of *Tubifex* and fish and with dehydrated food (Tetra Min). The next moults follow every 3-5 weeks. After the second moult the uropods are differentiated and the yolk is fully resorbed. After the sixth moult sexual differentiation is visible. The determination of the postlarval stages is based upon the number of moults after hatching (stage I, II, III etc.). For this study we collected intermoult animals from stage L until stage VI. For lack of sufficient amounts of animals, we did not collect the juveniles in stage V, but we collected the remaining animals in stage VI, after their sexual differentiation. For a description of the larval and postlarval development and the determination of the different stages of the moulting cycle, see Van Herp and Bellon-Humbert (1978).

For tests of biological activity we used freshwater crayfish of the species *Orconectes limosus*, obtained as described in Chapter II.

Light microscopy

The larvae and juveniles in the different developmental stages were decapitated and the rostral region of the cephalothorax was fixed, dehydrated, cleared and embedded in paraplast as described for the adult eyestalks in Chapter I. The immunocytochemical PAP staining on the serial sections (7 µm) was performed as described in Chapter I, with slight modification. To determine the optimum dilution and incubation time for the primary antiserum, several dilutions of the anti-*Astacus*-CHH serum were used and the incubation time was varied. An optimum dilution of 1/500 for the primary antiserum in combination with 72 h incubation time at 4°C, was applied for all investigated larval and postlarval stages.

Electron microscopy

For ultrastructural preparation total eyestalks of the stages L and I were collected in fixation fluid and the cuticle was gently stripped off. For the succeeding stages sinus glands were dissected. Fixation of eyestalks and sinus glands occurred in 2% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 8; 0.2 M sucrose) for 3 h at room temperature. After rinsing in buffer (16 h at 4°C) and dehydration in ethanol, the tissue was embedded in a mixture based on Epon 812 (Luft 1961) or in Spurr's resin (Spurr 1969). The eyestalks from stage L and I were sectioned at 1-2 µm. These semithin sections were studied after staining with toluidine blue according to Lynn (1965), to localize the region where the sinus gland was expected. From this area and from the sinus glands of the older postlarval stages ultrathin sections were cut with a Reichert OMU 2 ultramicrotome. Immunostaining of the ultrathin sections was performed as described in Chapter I, with the primary antiserum diluted 1/1000 in combination with an incubation time of 48 h at 4°C. The specificity of the immunocytochemical staining was tested as described in Chapter I. Part of the ultrathin sections was counterstained with 2% aqueous uranyl acetate (24 h at 4°C). The sections were examined in a Philips 300 electron microscope at 40 kV.

To our surprise only Epon embedded and not Spurr embedded material gave positive immunostaining. Adult sinus glands embedded in Spurr's resin also gave negative immunostaining. However, after removing the Spurr plastic out of semithin sections following the method described in Chapter III, we observed perfect immunoreactivity. We conclude that the negative results on the Spurr embedded ultrathin sections must be caused by some reversible interaction of this plastic with the antigenic determinants of the CHH material.

Morphometric analyses

The morphometric results of the light microscopic study are based on the analysis of both the left and the right eyestalk of 2 animals per developmental stage. For each eyestalk the number of immunopositive cells was counted and the largest and smallest diameters of the perikarya and nuclei were determined for all immunoreactive cells per eyestalk.

The morphometric results of the electron microscopic study are based on the analysis of 2 sinus glands from 2 specimens per developmental stage. For the quantitative work, the electron microscope was calibrated by use of a carbon replica grid (2160 lines per mm). The diameter of the immunopositive granules was measured on photographic prints by Hardware MOB equipment (Kontron Messgeräte GmbH).

Determination of hyperglycemic activity

To check the presence of a biologically active hyperglycemic factor in the eyestalk of the larvae, we injected small adult fresh water crayfish of the species *Orconectes limosus* with eyestalk extract of the larval stage (stage L). These animals were selected on little body weight, in order to minimize the injected dose of test extract. The injected dose of eyestalk equivalents was determined according to Leuven et al. (1982): $x/y \times 0.5$ eyestalk equivalents, in which x is the test animal's body weight and y the body weight of the donor. Lyophilized larval eyestalks were extracted in a physiological saline solution (modified Van Harreveld solution: 1017 mg NaCl; 40 mg KCl; 50 mg $MgCl_2.2H_2O$; 225 mg $CaCl_2$; 58 mg maleïne-acid; 476.6 mg HEPES in 100 cc distilled water; pH 7.5; 380 mOsm; room temperature). Blood samples (50 µl, in duplicate) were taken immediately before and 2 h after injection. The hemolymph was sucked in a calibrated capillary pipet, which was inserted between the coxa and the basis of the right cheliped. Controls were injected with saline. Blood glucose concentration was determined by the Glucose-GOD-Perid Methode (Boehringer, Mannheim).

RESULTS

Already in stage L, immediately after hatching, the four optic lobes are easily distinguishable. The location of the optic lobes resembles closely the situation found in the adult eyestalk, except for the medulla externa (ME) and the lamina ganglionaris (LG). The ME and LG in the larval and early postlarval stages have a dorso-lateral position with regard to the medulla interna (MI; Fig 1). During postlarval development the ME and LG shift to a medio-dorsal position as in the adult eyestalk. In all investigated stages the appearance of the neurosecretory cells, which are situated around the neuropil, is similar; the neuroblasts are of equal size and are oval shaped with a proportionally large nucleus enclosed by a small layer of cytoplasm (Figs 2,4).

Immunoreactivity to anti-CHH serum is found in the developing X-organ sinus gland complex of all investigated stages. The results of the morphometric study on the light microscopic level are summarized in Table 1 (part A). The immunopositive neuroblasts form a distinct group, located in a region latero-ventrally at the rostral side of the medulla terminalis (MT; Figs 2,4). During postlarval development, the number of immunostainable cells increases from approximately 20 in stage L to around 33 in stage VI, except for stage I where only half of the number reacts with the primary antiserum. During postlarval development the mean cellular and nuclear diameters remain constant. The axons of these cells form an immunopositive tract, which starts from the immunostained perikarya, passes in rostral direction through a part of the dorsal region of the MT and runs to the sinus gland area along the latero-dorsal side of the MT and MI (Fig 1). The axons end in the sinus gland where part of the axon terminals shows an immunopositive reaction (Figs 1,3).

TABLE 1

Morphometric data of the CHH-producing neurosecretory cells during larval and postlarval development of the crayfish Astacus leptodactylus.
A. Number and size of the immunoreactive cells determined by light microscopy.
B. Diameter of the immunopositive neurosecretory granules in the sinus gland determined by electron microscopy.

Developmental stage	A					B
	Number of immunoreactive cells per eyestalk	perikaryon		nucleus		diameter of immunoreactive granules (nm)
		largest diameter (µm)	smallest diameter (µm)	largest diameter (µm)	smallest diameter (µm)	
L	21 (±2.5)	18.7 (±0.3) (n^1=76)	13.8 (±0.3)	14.9 (±0.3) (n^1=76)	10.4 (±.0.3)	78 (±.3.9) (n^2=97)
I	9 (±0.5)	19 (±0.6) (n^1=31)	12.5 (±0.4)	14.3 (±0.7) (n^1=31)	10.2 (±.0.5)	91 (±.1.7) (n^2=514)
II	20 (±1.6)	18.7 (±0.4) (n^1=72)	13.4 (±0.3)	15.7 (±0.3) (n^1=72)	11.3 (±.0.3)	102 (±.3.2) (n^2=672)
III	23 (±1.3)	18.9 (±0.4) (n^1=88)	13.5 (±0.2)	14.7 (±0.3) (n^1=88)	10.6 (±.0.2)	116 (±.0.6) (n^2=987)
IV	33 (±2.1)	20.2 (±0.2) (n^1=126)	14.7 (±0.2)	14.3 (±0.2) (n^1=126)	11 (±.0.2)	123 (±.0.9) (n^2=1253)
VI	33 (±7)	19.7 (±0.4) (n^1=120)	14.2 (±0.4)	14.9 (±0.4) (n^1=120)	10.9 (±.0.3)	121 (±.0.6) (n^2=782)

A. Results obtained after studying 2 animals per developmental stage (2 eyestalks per animal); means ±S.E.M.; n^1 = number of measured cells.
B. Results obtained after studying 2 animals per developmental stage (1 sinus gland per animal); means ±S.E.M.; n^2 = number of measured granules.

The morphometric results of the larval and postlarval sinus glands after PAP staining on the ultrastructural level are summarized in Table 1 (part B). In the sinus glands of all investigated developing stages an immunopositive reaction is observed in neurosecretory granules of distinct axon terminals. Until stage I the immunoreactive granules appear rather "fuzzy" (Figs 5A,5B). During further development the immunostaining results in a more distinct appearance of the granules (Figs 6A,6B). The mean diameter of the immunopositive neurosecretory granules increases from around 78 nm in stage L to around 120 nm in stage VI. The counterstained ultrathin sections show other types of axon terminals which do not react with the primary antiserum (Figs 5B,6B). The size and electron density of these granules differ from those of the immunoreactive granules.

The incubations performed to test the specificity of the immunoreaction reveal no immunopositive results.

Injection of larval eyestalk extract (from stage L) into small adult *Orconectes limosus*, results in a marked rise of the hemolymph glucose concentration 2 h after injection. Before injection, the blood glucose level (±S.E.M.) for the 12 test animals was 2.28 (±1.24) mg per 100 ml hemolymph. Two hours after injection, the blood glucose level of 3 controls stays 2.4 (±0.5). Injection of larval eyestalk extract in 9 animals, resulted in a rise to 20.5 (±6.3) mg glucose per 100 ml hemolymph.

DISCUSSION

During the larval and postlarval development of the crayfish *Astacus leptodactylus*, an immunopositive reaction for the anti-*Astacus*-CHH serum is found in neurosecretory cells of the MTGX, in the MTGX-sinus gland tract and in part of the axon terminals of the sinus gland. The location of these immunopositive regions in the larval and juvenile eyestalks is similar to the location of the CHH system in adult decapod crustaceans as determined by the same staining method (Chapters I,II; Jaros and Keller 1979; Van Herp et al., in press).

After immunocytochemical investigation of the sinus gland of the larval and postlarval stages at the electron microscopic level, we find an immunopositive reaction in neurosecretory granules present in axon terminals. As judged by the diameter of these immunopositive granules, they belong to the same type in each developmental stage.

Furthermore, injection of larval eyestalk extract causes a marked rise of the blood glucose concentration in the crayfish *Orconectes limosus*. This implicates that a hyperglycemic factor is already present in the eyestalks directly after hatching. This indicates that this factor is already active in the larvae and juveniles, although experimental confirmation is necessary.

During the development of the juvenile eyestalks the number of cells reacting with the primary antiserum increases. It is possible that during postlarval development neuroblasts differentiate into immunoreactive cells. The formation of CHH cells by means of mitotic division does not seem likely as we never observed any mitotic activity in the MTGX region of the juvenile eyestalks. It is also possible that already in stage L, all CHH cells are present but that part of them do not yet exhibit synthetic activity or that not all cells are synthetically active at the same time. Since in stage I the number of immunoreactive cells is only half of the number found in the preceeding larval stage, the second possibility seems more likely. This difference points to a higher level of secretory activity during stage L than during the following stage. This higher activity in stage L might be connected with metamorphosis of the larvae and with the circumstance that the larvae are metabolizing their vitellin

stores. These vitellin stores might be the first targets for the hyperglycemic hormone. During stage I the vitellin is fully resorbed and the animals search for food. This switch in food supply may explain the observed decrease in number of synthetically active CHH cells.

The immunopositive reaction of the granules in sinus glands of the larval and first postlarval stage appears rather "fuzzy" in comparison with the more distinct immunoreaction observed in the older stages. This may be due to inadequate fixation of the granules caused by slow penetration of the fixation liquid, since for the first stages, we had to fix the whole eyestalks. In the course of development the size of the immunopositive granules increases. In stage VI the diameter of the CHH granules is around 121 nm, while in the adult sinus gland it is aroud 143 nm (Chapter I). This implicates further development of the CHH system.

In the ultrathin sections poststained with uranyl acetate, we also observed granules that were smaller in size and lower in electron density than the CHH granules and that never exhibited an immunopositive reaction. They apparently do not originate from CHH cells.

The results of this study show that no conclusions on the appearance of neurosecretory products during larval and postlarval development may be drawn, that are exclusively based on the diameter and shape of the neurosecretory granules. Further identification, as i.e. by means of immunocytochemistry, is necessary.

ABBREVIATIONS

BM	basement membrane
H	hemolymph
LG	lamina ganglionaris
ME	medulla externa
MI	medulla interna
MT	medulla terminalis
MTGX	medulla terminalis ganglionic X-organ
N	nucleus of glial cell
O	ommatidia
SG	sinus gland
T	tract

LEGENDS

Fig. 1: Stage I: Immunopositive reaction in the sinus gland and the tract. Bar represents 100 µm.

Fig. 2: Stage L: Immunoreactive cells in the MTGX. Bar represents 10 µm.

Fig. 3: Stage I: Distinct immunopositive regions in the sinus gland (arrow). Bar represents 10 µm.

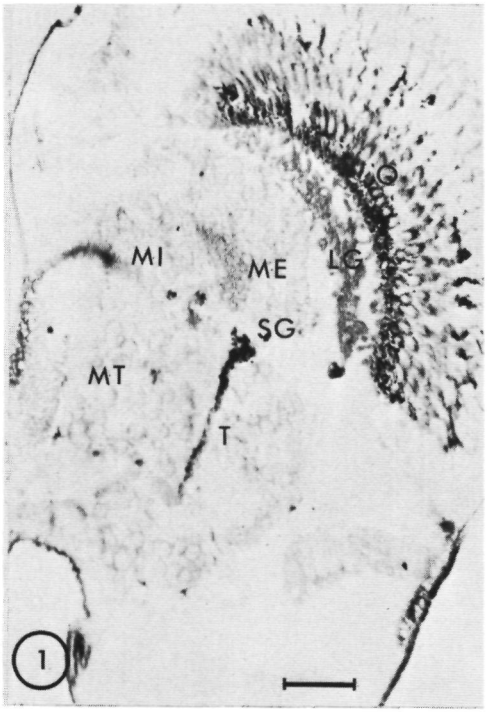
Fig. 4: Stage IV: Immunoreactive cells in the MTGX. Bar represents 10 µm.

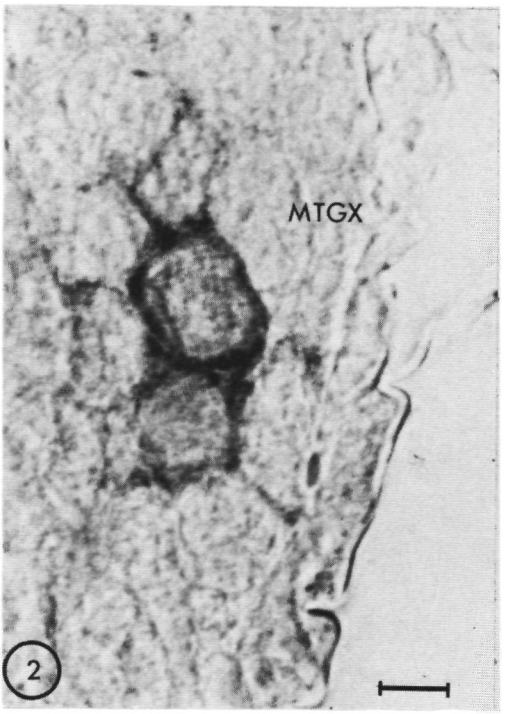
Fig. 5A: Stage I: Immunopositive axon terminal in the sinus gland filled with immunoreactive granules (arrow). Compare with succeeding section (Fig 5B). Bar represents 500 nm.

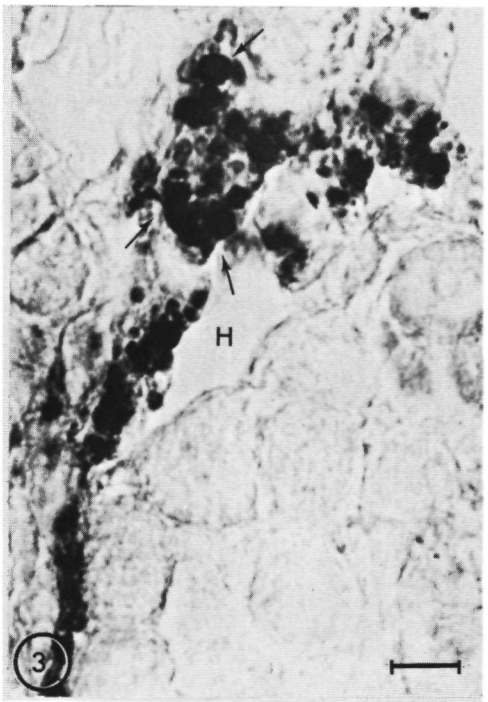
Fig. 5B: Stage I: Section counterstained with uranyl acetate, showing the same region as presented in Fig 5A. Note axon terminal containing another type of neurosecretory granules (arrow). Bar represents 500 nm.

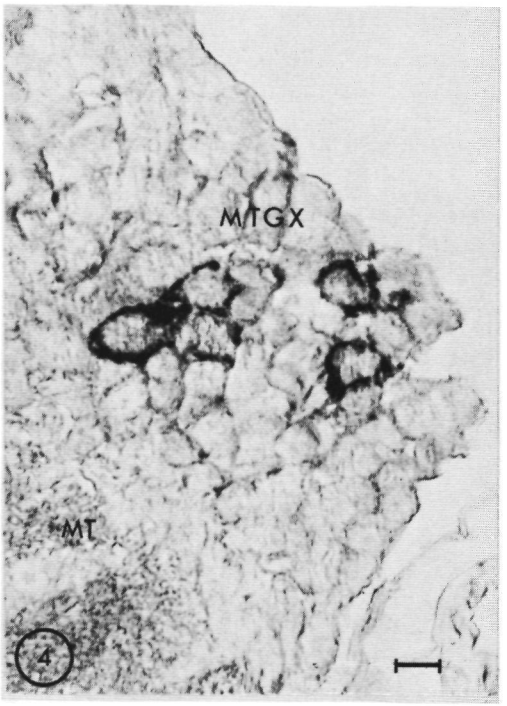
Fig. 6A: Stage IV: Immunopositive axon terminals in the sinus gland. Compare with succeeding section (Fig 6B). Bar represents 1 µm.

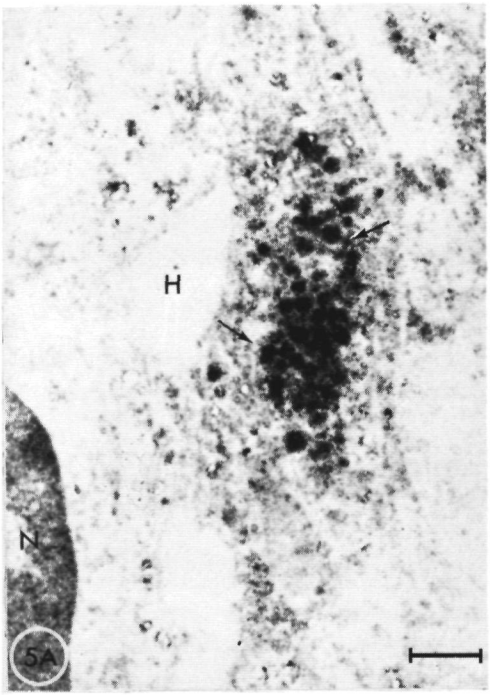
Fig. 6B: Stage IV: Section counterstained with uranyl acetate, showing the same region as presented in Fig 6A. Bar represents 1 µm.

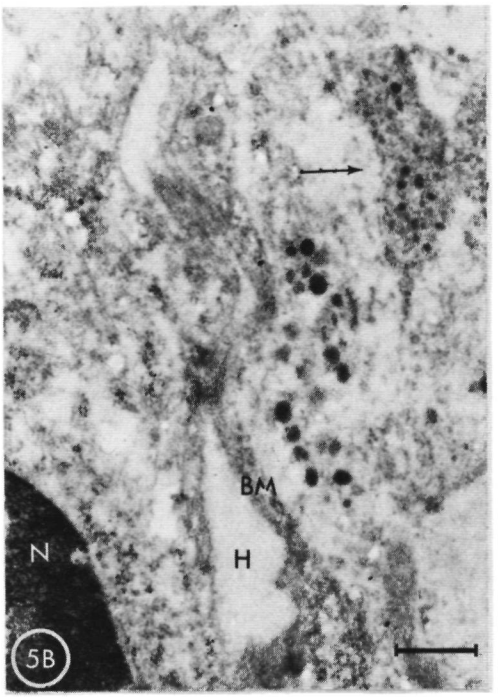

MI
ME
LG
SG
MT
T
1

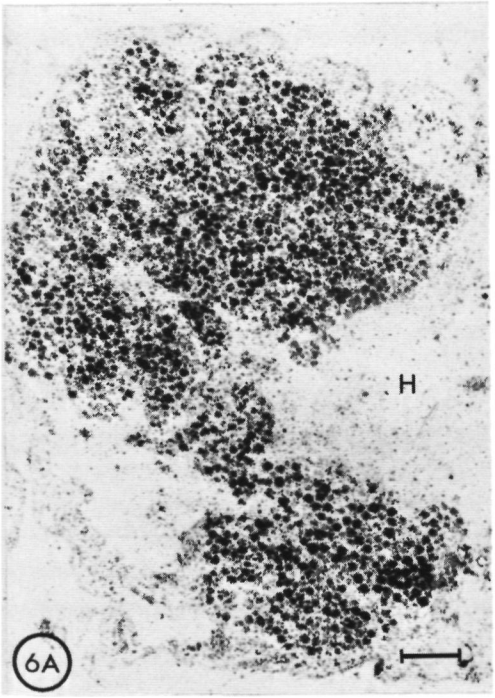

MTGX
2

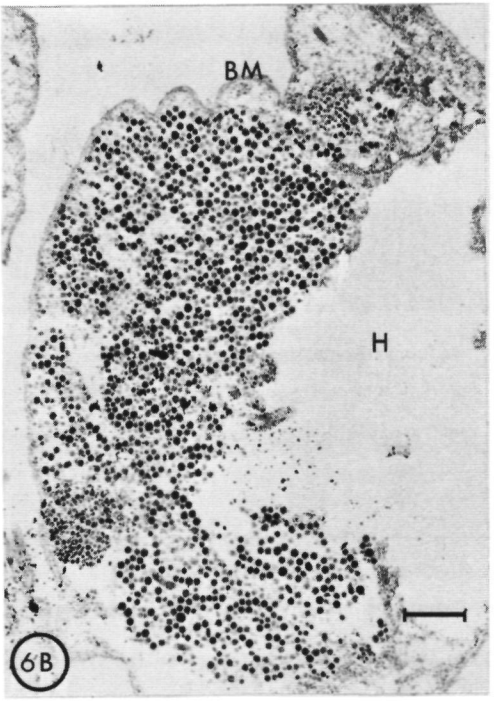

H
3


MTGX
MT
4


H
N
5A


BM
N
H
5B


H
6A


BM
H
6B

CHAPTER VII

STRUCTURE AND INNERVATION OF THE CRUSTACEAN HYPERGLYCEMIC HORMONE-PRODUCING CELLS IN THE EYESTALK OF THE CRAYFISH ASTACUS LEPTODACTYLUS, AS STUDIED WITH LUCIFER YELLOW AND ELECTRON MICROSCOPY

Gorgels-Kallen (1985),
Cell and Tissue Research, in press.
Presented at the VIIIth
Réunion des Carcinologistes de Langue Française,
Liège (29.08-02.09.1983):
Gorgels-Kallen (1984a).

SUMMARY

Injection of the fluorescent dye Lucifer Yellow into single crustacean hyperglycemic hormone (CHH)-producing cells, permits tracing of the total shape of these neurosecretory cells in the eyestalk of the crayfish ***Astacus leptodactylus***. Highly fluorescent perikarya pass into axons that can be followed by the fluorescent label until the neurohemal region, the sinus gland. The proximal part of that axon exhibits extensive branching into the neuropil of the medulla terminalis. Electron microscopic investigations reveal synaptic input on these axon ramifications.

INTRODUCTION

Cobalt iontophoresis by means of backfilling through the cut base of the sinus gland, or injection of single cells in the X-organ, have been useful in clarifying the topography of the neurosecretory system in the crustacean eyestalk (Andrew et al. 1978; Andrew and Saleuddin 1978; Jaros 1978). These studies showed extensive branching of the axons of the neurosecretory X-organ cells in the medulla terminalis neuropil. Glantz et al. (1983) observed similar axonal branching after injection of the fluorescent tracer Lucifer Yellow into single X-organ cells. Andrew and Saleuddin (1978) described synaptic input on the observed arborizations.

Since we are able to localize the CHH-producing cells by use of an anti-*Astacus*-CHH serum with the PAP method (Chapter I), we studied the microstructure of this system in more detail, to detect possible relationships with other neuronal elements. We tried to vizualize single CHH-producing cells by micro-electrophoretic injection of the fluorescent dye Lucifer Yellow CH and we studied the observed ramifications of the axons in the medulla terminalis. The region where the CHH axons travers the medulla terminalis neuropil, was studied at the ultrastructural level.

MATERIALS AND METHODS

Animals

Crayfish of the species ***Astacus leptodactylus*** were obtained commercially and kept in the laboratory as described in Chapter I. For the experiments, adult male crayfish were used, in stage C of their moulting cycle (Drach 1944).

Preparation of the CHH cells for injection

Eyestalks were ablated and immediately brought into a physiological saline solution (modified Van Harreveld solution, described in Chapter VI). By use of a dissection stereo microscope, the cuticle on the ventral side of the eyestalk was removed and the underlying hypodermis, muscles and connective tissue were gently removed until the medulla terminalis ganglionic X-organ (MTGX) and the medulla terminalis neuropil became visible. The CHH cells can be easily detected due to their size (diameter around 50 µm) and appearance: they are seen as opaque grey-blueish dots situated superficially in the

latero-ventral region of the MTGX (for a picture of the location of the CHH cells see Chapter II).

Micro-iontophoresis with Lucifer Yellow

Single CHH cells were injected with a 3% aqueous solution of the fluorescent tracer Lucifer Yellow CH (Sigma). The micro-electrodes (glass capillaries with inner filament, TW 150, diameter 3 mm, tip diameter around 2 µm, W.P. Instruments Inc.) were filled with dye via the large end of the electrode. The resistance of filled electrodes varied around 100 MΩ. The tip of the micro-electrode was inserted in a CHH cell by use of a micro-manipulator and the dye injected by passing microcurrent hyperpolarizing pulses of 5-10 nA with a duration of 400 msec and a frequency of 1 Hz. During injection the action potential and cell potential were monitored by use of a Tektronix oscilloscope (type RM 561 A). Injection times were varied between 10 min and several hours. The technique of Lucifer Yellow iontophoresis is extensively described by Stewart (1978) and used in our study without further modifications.

Fluorescence microscopy of injected preparations

Wholemount eyestalks were examined immediately after injection. Some preparations were fixed for 1 h in 0.1 M sodium phosphate buffer (pH 7.4) containing 4% formaldehyde as fixative. Eyestalks were dehydrated in a graded aceton series and embedded in Spurr's resin (Spurr 1969). Semithin sections (1.5 µm) were cut on a Reichert OM U2 ultramicrotome and mounted on glass slides by means of a 1% aqueous gelatine solution. Both wholemounts and sections were observed with a Leitz Orthoplan universal microscope equipped with a Phloemopak fluorescence epi-illuminator, an Osram HBO 200 W lamp and a 2 mm BG-12 filter. Photomicrographs were taken on Kodak Tri-X Pan film (400 ASA).

Immunocytochemistry

To verify that the injected cell is a CHH cell, semithin Spurr embedded sections of injected preparations were immunostained following the procedure described in Chapter III.

In order to visualize the CHH material in the arborizations of the CHH axons in paraplast embedded non-injected eyestalks, several specimens were fixed in Bouin-Hollande fluid containing 10% of a saturated aqueous solution of sublimate. Dehydration, embedding in paraplast and immunostaining of the 7 µm sections were performed as described in Chapter I, with a primary antiserum dilution of 1/400.

Electron microscopy of the axon arborizations

The medulla terminalis was dissected, fixed and embedded in Spurr's resin as described in Chapter III. Ultrathin sections were cut and poststained and the morphometric analyses of the ultrastructural pictures were performed following the prescription given in Chapter III.

RESULTS

Injection of Lucifer Yellow into single CHH cells reveals a clearly distinguishable perikaryon with a highly fluorescent nucleus (Figs 1,3). The perikaryon gives rise to an axon that runs from the rostral latero-ventral part of the MTGX through the ventral part of the medulla terminalis neuropil to the external side of the eyestalk. Here it proceeds distally until it splits up into several axon terminals in the sinus gland. The proximal part of the axon exhibits extensive branching into the rostral latero-ventral and latero-dorsal part of the medulla terminalis (Figs 1,7). These arborizations show abundant varicosities. These are also visible, although to a lesser extent, in the proximal part of the main axon (Fig 2). Both the cell body and the axon are brightly stained after 10 min of marking. Longer injection times do not result in more satisfying results. Occasionally, labeling of the axon terminals in the sinus gland was observed. Injected cells sometimes may loose all dye in a few minutes. We prevented this by fixation of the marked eyestalk immediately after injection. The fixation gave good preservation of the tissue and did not affect fluorescence.

Immunostaining on semithin sections of injected eyestalk specimens reveals that the Lucifer Yellow labeled CHH cells show a positive reaction for the primary antiserum, which is only slightly weaker than the immunoreaction in the surrounding non-labeled cells. The injected CHH cells still exhibit faint fluorescence after immunostaining (Figs 4A-B).

In paraplast embedded 7 µm sections the ramifications of the CHH axons show a positive immunoreaction (Figs 5, 6).

Electron microscopic investigation of the neuropil area in the medulla terminalis (framed area in Fig 5), shows a compact tract almost exclusively composed of CHH axons. The center of these axons is filled with numerous microtubules. At its periphery, neurosecretory CHH granules with a diameter of around 134 nm are found, together with mitochondria and irregularly shaped electron translucent vesicles. Glial cytoplasmic ramifications are mostly absent (Fig 8). At the periphery of the proximal part of the CHH tract and deeper into the neuropil of the medulla terminalis many fine axon processes are found with a diameter varying from 0.5 to 2.5 µm (Figs 8,11). Deeper into the neuropil they are densely packed with neurosecretory granules similar to the granules which characterise the CHH axons. Furthermore, mitochondria are abundant in these axon branches while little or no microtubules are observed (Fig 9). Close to these CHH axon ramifications a considerable number of neuronal processes is found. They are closely packed with two types of inclusions. One type, with a diameter of about 67 nm, is surrounded by a halo and is moderately dense; the second type is an electron-lucent vesicle with a diameter of around 36 nm (Figs 8-11). The neuronal processes are furthermore characterized by many large mitochondria. In addition, they show synaptic contacts with the CHH branches. The observed synapses all share the following features: a. the adjacent CHH and neuronal processes are separated by a 20 nm synaptic cleft; b. the neuronal processes contain synaptic vesicles associated with the synaptic cleft, which resemble the clear vesicles in the presynaptic regions; c. the CHH processes contain a thick synaptic density contiguous with the synaptic cleft (Fig 10). Indications that one presynaptic process contacts more than one CHH branch are frequently observed (Fig 11). Occasionally, synaptic contacts are found on the main CHH axon (Fig 11).

In vivo injection with Lucifer Yellow of single CHH cells in the eyestalk of the crayfish ***Astacus leptodactylus***, results in a labeled perikaryon passing into an axon which traverses the medulla terminalis neuropil and runs to the sinus gland, where it splits up into several axon terminals. This appearance is in concordance with the description of the location of the CHH system as visualized immunocytochemically by use of an anti-*Astacus*-CHH serum (Van Herp and Van Buggenum; Chapter I).

Many fine processes originate in the proximal part of the axon and protrude into the medulla terminalis neuropil. Comparable arborizations of axons, originating from unidentified neurosecretory X-organ cells, into the medulla terminalis also have been described by Andrew et al. (1978) and Jaros (1978), after cobalt iontophoresis, and by Glantz et al. (1983) after tracing with Lucifer Yellow. Our ultrastructural observations show that the arborizations of the CHH axons are closely packed with granules but that microtubules, indicating transport of neurosecretory material, are scarcely found. Furthermore, many varicosities are observed. Indications for release of neurosecretory material via the ramifications, like exocytotic profiles or fine processes bordering blood sinuses or intracellular cavities, were not observed. Axon varicosities are also described by Glantz et al. (1983) and by Steel (1977). The latter suggests that these axon swellings might serve as a reservoir for neurosecretory material.

Many synaptic figures are observed between the described fine CHH branches and the neuronal processes, that contain haloed dense-cored granules and clear vesicles. In these neuronal processes, synaptic vesicles are present in association with the synaptic cleft while the CHH processes contain a thick synaptic density. This indicates that the CHH branches can be considered postsynaptic elements. Synaptic input on neurosecretory axons is also described by Shivers (1967) for the crayfish ***Orconectes nais*** and by Andrew and Saleuddin (1978) for the crayfish ***Orconectes virilis***. These authors suggest the presence of aminergic products as neuromodulators in the presynaptic axons. The possible role of biogenic amines and cyclic nucleotides in the release of neurosecretory substances from the sinus gland of ***Astacus leptodactylus*** has been investigated previously in our laboratory. With the Falck-Hillarp method Strolenberg (1979) obtained fluorescence in the optic nerve and all optic ganglia except the lamina ganglionaris. Strolenberg and Van Herp (1977) and Van Herp and Strolenberg (1980) studied the possible role of serotonin and d-c-AMP in the release of CHH. Injection of these substances into the crayfish hemolymph evoked an increase in the number of exocytoses of the type V granules (CHH granules) in the sinus gland of ***Astacus leptodactylus***, and was followed by hyperglycemia in the blood. Comparable results were obtained by Keller and Beyer (1968) for the crayfish ***Orconectes limosus*** and by Martin (1978) for the isopod ***Porcellio dilatatus***. An immunocytochemical study on the prawn ***Palaemon serratus*** revealed a positive reaction with anti-serotonin (Bellon-Humbert et al., submitted). However, the idea that transmission of information by neurons takes place only by means of non-peptidergic substances has been superseded. Recent studies on neurotransmitters revealed the neuromodulating function of peptides (e.g. Polak and Bloom 1978; Emson 1979; Buys and Swaab 1979; Adams and O'Shea 1983). In crustaceans, immunocytochemical investigations revealed a positive reaction in the eyestalk for anti-enkephalin and anti-substance P (Mancillas et al. 1981; Jaros and Keller 1983; Jaros, in prep; Jaros et al, in prep). for anti-neurophysin I and anti-vasopressin (Van Herp and Bellon-Humbert 1982), for anti-somatostatin (Martin and Dubois 1981) and for anti-FMRFamide (Jacobs and Van Herp 1984). In our laboratory, an immunocytochemical study of the eyestalk of ***Astacus leptodactylus*** with antisera against several neuropeptides

revealed immunopositive reactions in perikarya and axons, including many axon processes in the medulla terminalis neuropil (Van Deijnen et al. 1984; Van Deijnen et al., in press). More detailed information about the chemical nature of the substance(s) in the present described neuronal processes, is required because they probably represent the only neuronal input to the CHH system. We never observed synapses or synapse-like structures on the perikaryon, the axon hillock or the axon terminals of the CHH cells. An immunocytochemical study of this important neuropil region with different antisera is in progress.

The presence of Lucifer Yellow does not lead to noticeable changes in immunoreactivity of the CHH cells, since the immunopositive reaction to PAP staining of the injected cell is only slightly reduced. This implies that, if Lucifer Yellow would combine with CHH material, this does not affect seriously the antigenic determinants of the CHH molecule for the anti-*Astacus*-CCH serum. Contrastingly, the fluorescence of the injected cell is noticeably reduced after PAP staining. This may be caused by the etching and incubation procedures necessary for immunocytochemical staining.

ABBREVIATIONS

CHH	CHH axon process
D	dorsal side
LG	lamina ganglionaris
ME	medulla externa
MI	medulla interna
MT	medulla terminalis
MTGX	medulla terminalis ganglionic X-organ
NO	optic nerve
O	ommatidia
SG	sinus gland
T	tract
V	ventral side

LEGENDS

Fig. 1: Overall view of a CHH cell injected with Lucifer Yellow. Encircled area indicates the main region with axon ramifications. Bar represents 50 µm.

Fig. 2: Detail of CHH axon branches filled with Lucifer Yellow. Arrows indicate varicosities. Bar represents 5 µm.

Fig. 3: CHH cell injected with Lucifer Yellow. Note intens staining of the nucleus. Bar represents 10 µm.

Figs 4A,B: Semithin section of a Lucifer Yellow injected CHH cell (arrow), still showing fluorescence (Fig 4A) after immunocytochemical staining (Fig 4B). Bar represents 50 µm.

Fig. 5: Paraplast embedded section showing immunostaining of the tract and the CHH axon branches in the medulla terminalis neuropil (framed area). Bar represents 100 µm.

Fig. 6: Detail of the fine CHH axon ramifications in the medulla terminalis neuropil after PAP staining (arrows). Bar represents 5 µm.

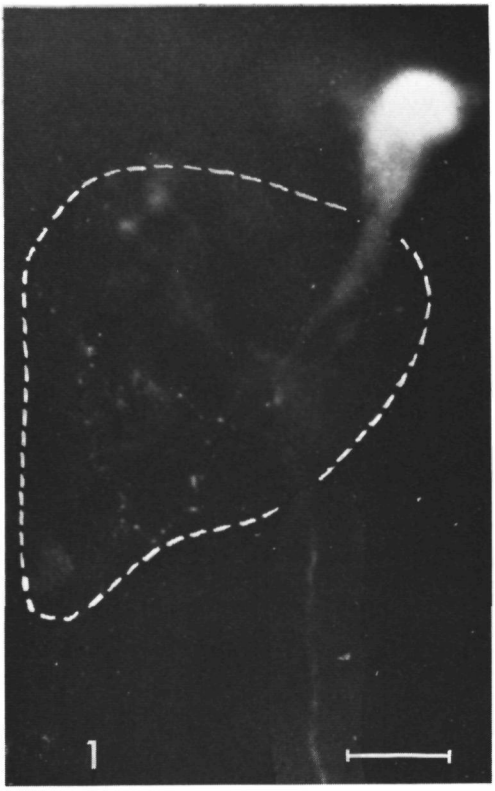
Fig. 7: Diagram of the shape of a CHH cell as visualized after injection with Lucifer Yellow.

Fig. 8: Survey of the CHH tract and the proximal part of the axon ramifications (arrows). Bar represents 5 µm.

Fig. 9: Ultrastructure of the distal part of a CHH axon branch (arrow). Note the densely packed CHH granules. Bar represents 1 µm.

Fig. 10: Ultrastructure of the synaptic contact on a CHH axon branch (arrow). Bar represents 500 nm.

Fig. 11: View of a presynaptic axon making synaptic contact with more than one CHH process (arrows) and a main CHH axon (big arrow). Bar represents 1 µm.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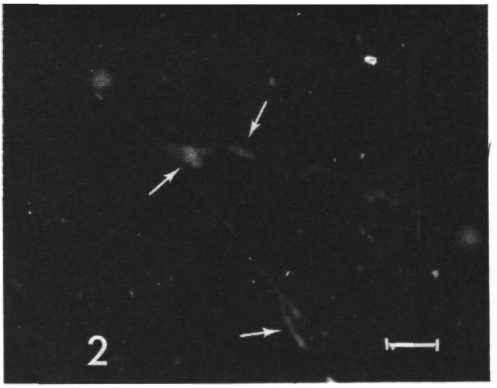
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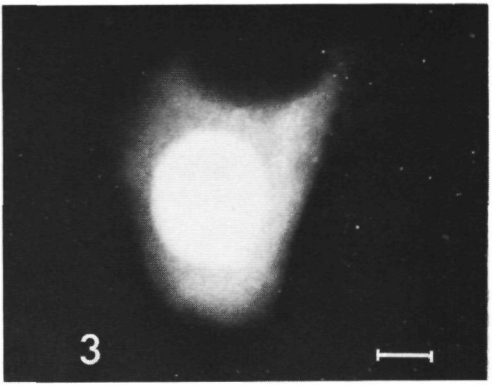
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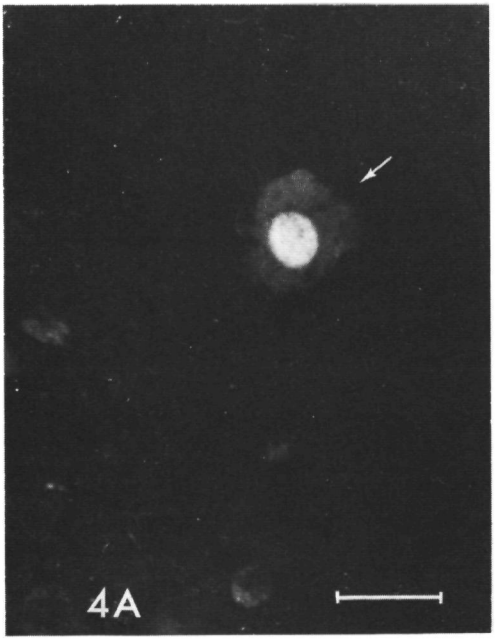
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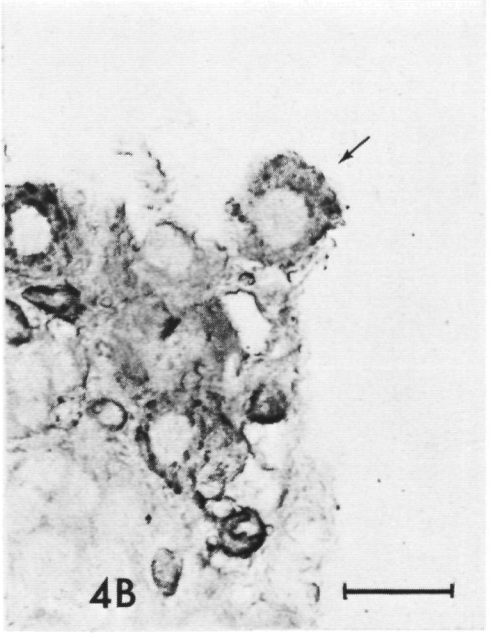
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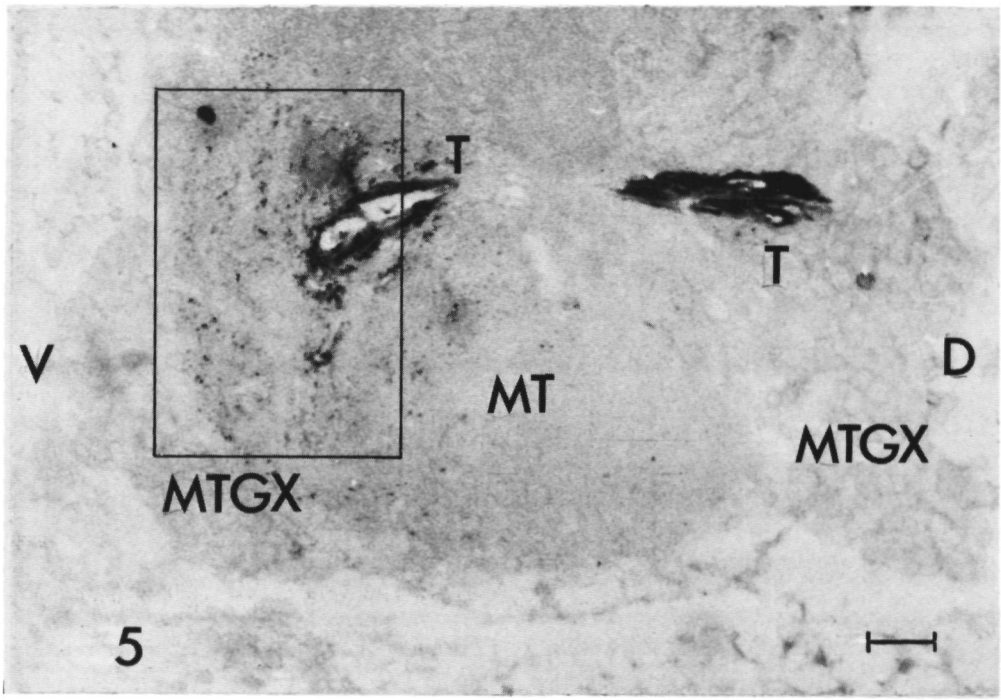
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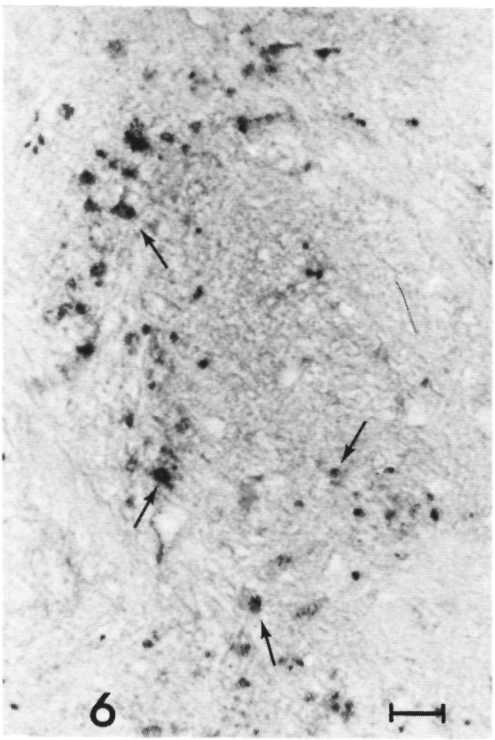
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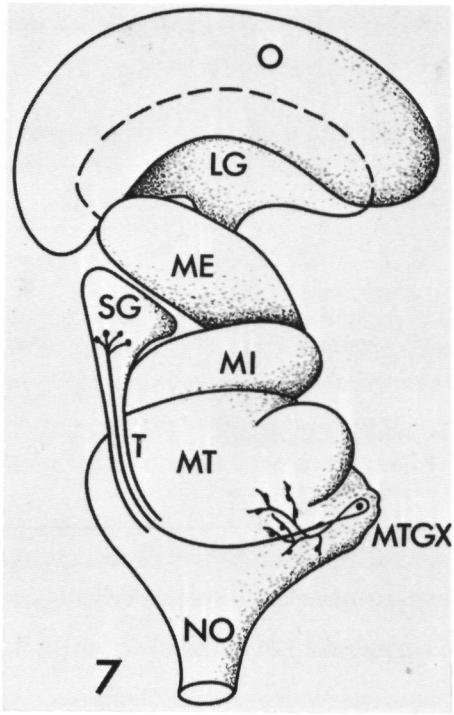
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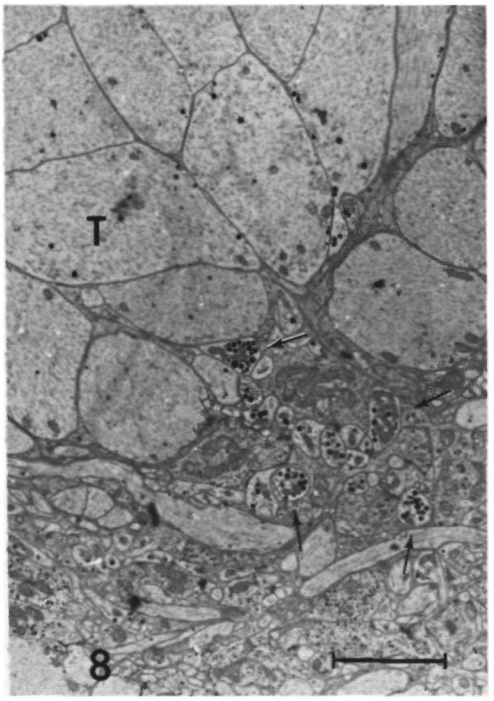
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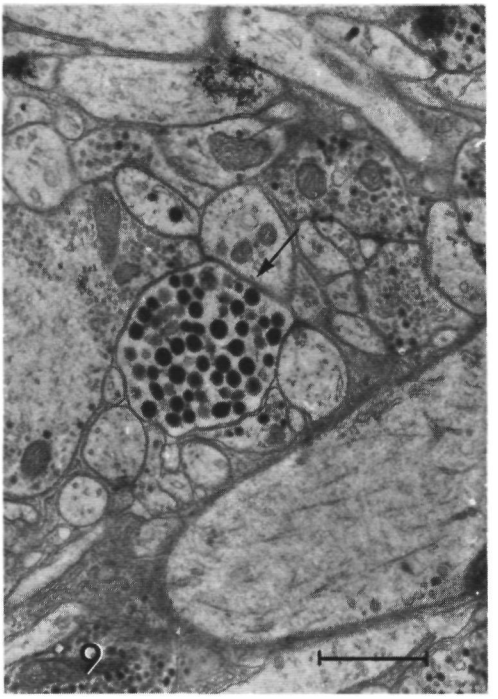
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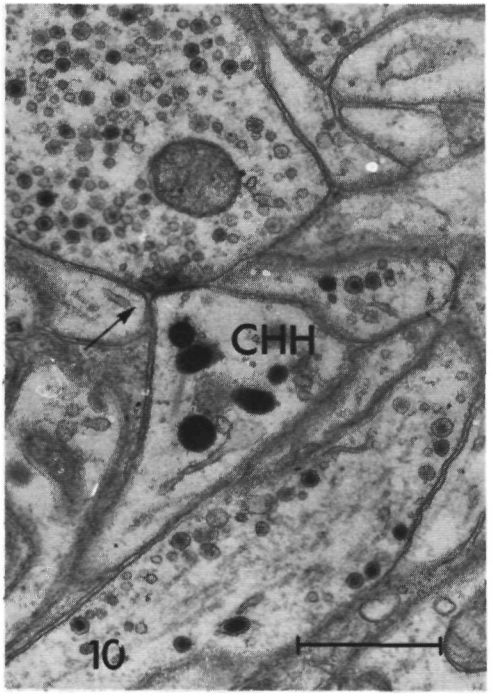
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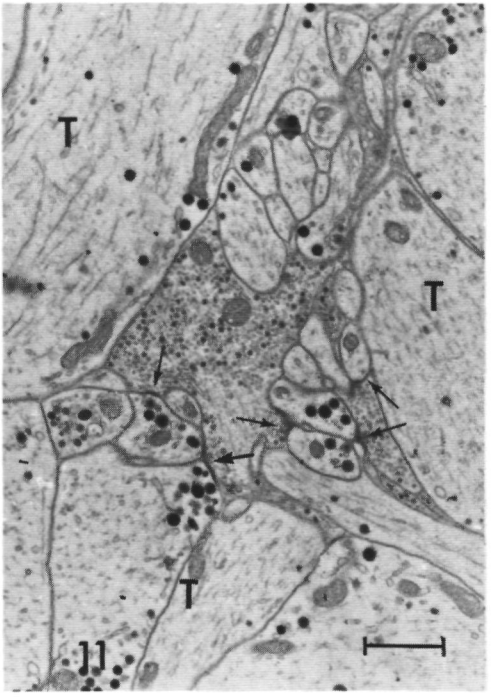
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CHAPTER VIII

BIOCHEMICAL ANALYSES OF THE CRUSTACEAN HYPERGLYCEMIC HORMONE OF THE CRAYFISH ASTACUS LEPTODACTYLUS

General and Comparative Endocrinology, in prep.
Presented at the
International CNRS Symposium in Strassbourg, (29.08-02.09.1983):
(Gorgels-Kallen and Van Herp 1983),and at the VIIIth
Réunion des Carcinologistes de Langue Française,
Liège, (29.08-02.09.1983): Gorgels-Kallen (1984b).

SUMMARY

A biochemical analysis was made of the structure of the crustacean hyperglycemic hormone (CHH) of the crayfish ***Astacus leptodactylus***, as present in the CHH-producing perikarya, in the axonal tract and in the sinus gland, respectively. Hyperglycemic products were analysed by polyacrylamide gel electrophoresis (PAGE and SDS-PAGE) and high pressure liquid chromatography (HPLC) in combination with a dotting immunobinding assay (DIA) and a bioassay for hyperglycemic activity.

After electrophoretic analyses the predominant biologically as well as immunologically detectable product present in all parts of the cell has an apparent molecular radius of approximately 7000 D. In the perikarya extract a second factor with lower electrophoretic mobility was found, which may represent the prohormone or precursor of CHH.

The analyses by means of HPLC showed two predominant immunopositive peaks with an elution time of 28-29 min and 52-54 min, respectively. Electrophoresis of these fractions indicates a molecular radius of 7000 D for both HPLC peaks.

INTRODUCTION

The crustacean hyperglycemic hormone (CHH) is the biochemically most studied metabolic hormone in decapod crustaceans. Early reports have characterized the CHH as a protein with a molecular weight of 6000-7000 D (Kleinholz 1966; Kleinholz et al., 1967; Keller 1968; Kleinholz and Keller 1973). The amino acid composition of CHH was elucidated for the crab ***Cancer magister*** (Kleinholz 1975), the crab ***Carcinus maenas*** (Keller and Wunderer 1978), the crayfish ***Orconectes limosus*** (Keller 1981) and the land crab ***Cardisoma carnifex*** (Newcomb 1983).

Studies on the electrophoretic mobility of hyperglycemic molecules of various decapod species revealed remarkable differences, which indicate a diversity in the structure of these neuropeptides (Kleinholz and Keller 1973; Keller 1977). Furthermore, species specificity has been reported for bioactivity of this hormone by Keller (1968, 1969) Kleinholz and Keller (1973) and Leuven et al. (1982). Several authors reported different molecular forms of the hyperglycemic material for one species. For molecular weight, Skorkowski et al. (1977) reported two molecular forms for the shrimp ***Crangon crangon*** (20500 and 7300 D). Newcomb (1983) and Stuenkel (1983) described the occurrence of at least two different products containing hyperglycemic activity for the land crab ***Cardisoma carnifex***. Van Wormhoudt et al. (1984a,b) reported the presence of three bioactive CHH fractions for the prawn ***Palaemon serratus*** (20000, 7000 and 3000 D). On the contrary, Trausch and Bauchau (1981) found that hyperglycemic activity was restricted to a small peptide for the lobster ***Homarus americanus*** (1500 D).

Until now, the morphology of the CHH-producing system of the crayfish ***Astacus leptodactylus*** has been described in detail (Van Herp and Van Buggenum 1979; Chapter I to VII). However, little is known about the biochemical properties of the ***Astacus***-CHH molecule in the neurosecretory CHH pathway. The study of the ***Astacus*** CHH-producing system gave morphological indications for molecular transformation of the CHH during its transport from the perikarya to the sinus gland. Electron microscopic investigations revealed an increase in mean diameter together with a decrease in electron density of the CHH-containing neurosecretory granules upon transportation from their sites of production in the cell body to the site of release in the axon terminals (Chapter III). Differences in the electron density of secretory granules have

frequently been interpreted as reflecting biochemial changes in the nature of the granule content. This process is generally referred as maturation of the elementary granules (e.g. Berlind 1977). The observed changes in diameter and electron density of the CHH granules may form additional morphological indications for putative biochemical changes in the nature of the hyperglycemic material.

In this respect, we made a study of the hyperglycemic material present in the CHH-producing perikarya, the tract and the sinus gland, respectively. In order to identify the *Astacus*-CHH and to compare the results with the previous work mentioned above, we applied both PAGE and SDS-PAGE. Furthermore analyses were performed by HPLC. CHH material was identified by analysing the obtained gel and HPLC fractions via immunochemical detection and a bioassay for hyperglycemic activity.

MATERIALS AND METHODS

Animals

Crayfish of the species ***Astacus leptodactylus*** were obtained commercially and kept in the laboratory as described in Chapter I. In our tests for bioactivity we also included crayfish of the species ***Orconectes limosus.*** They were obtained as mentioned in Chapter II.
All experiments were carried out with normally fed adult male crayfish, in stage C of the moulting cycle (Drach 1944).

Tissue sampling

Eyestalks of the species ***Astacus leptodactylus*** were ablated and brought into distilled water. Using a dissection stereo microscope, the cuticle, underlying hypodermis, muscles and connective tissue were removed to expose the CHH-producing neurosecretory system (about 90% of the dissected cells represented CHH cells). The X-organ sinus gland tract and the sinus gland were dissected and collected separately in glass/glass potter homogenizers containing ice-chilled distilled water for PAGE or ice-chilled 0.1 N HCl (for HPLC). Tissues were homogenized and centrifuged. For electrophoreses the supernatants thus obtained were lyophilized and stored at -20°C. For HPLC analyses freshly prepared supernatants were chromatographed.

Analytical techniques

Separation of the samples was performed using either the technique of polyacrylamide gel electrophoresis (PAGE and SDS-PAGE) deduced from the methods of Davis (1964) and Laemmli (1970), respectively, or by reversed phase high pressure liquid chromatography (HPLC) applied as described by Martens et al. (1980).

PAGE was performed using a 0.75 mm thick slabgel containing a 4% stacking gel, pH 6.7 and a 7% separating gel, pH 8.9. Electrophoresis was carried out in a Bio-Rad Protean cell 16 CM , at 125 V for migration through the stacking gel and 250 V for separation in the 7% gel. The electrophores were

stopped after 3 to 5 h when the Bromophenol Blue front in the separation gel reached 12 cm. Then parts of the gels were either stained with Coomassie Brilliant Blue (CBB) or with a silver staining procedure as described by Morrissey (1981). Other slots were subdivided in 5 mm slices which were eluted for 24 h in ice-cold distilled water. The eluants were lyophilized and stored at -20°C until further analyses.

HPLC (reversed phase) was performed on a Spectra Physics high performance liquid chromatograph (model SP 8000) equipped with a manual injector and a ternary gradient system. The stainless steel column (250 × 4.6 mm) was packed with Spherisorb 10 ODS (Chrompack B.V.). The linear gradient was established with Solvent A (0.5 M formic acid, 0.14 M pyridine; pH 3) and Solvent B (1-propanol). The material was eluted with a flow rate of 2 ml/min and 0.5 ml fractions were collected, lyophilized and stored at -20°until further analyses.

Immuno- or bioactive fraction(s) of the sinus gland extract obtained after PAGE were analysed by SDS electrophoresis. Lyophilized material was dissolved in buffer containing 2-mercaptoethanol and boiled for 2 min. Electrophoresis was carried out by use of a 3% stacking gel (0.1% SDS, pH 6.8) and a 15% separating gel (0.1% SDS, pH 8.8) at 10 mA/cm gel for the 3% gel and 20 mA/cm gel for the 15% gel. Under these conditions the migration front in the separation gel reached 12 cm after about 3 h. Calibration proteins: bovine serum albumine (Serva: 67000 D); glutamate dehydrogenase (Serva: 53000 D); ferritin (Boehringer: 18500 D); myoglobin (Serva 17000 D); cytochrome c (Boehringer: 12500 D); ACTH (Serva: 4500 D); insulin β (Serva: 3495 D); mellitin (Serva: 2840 D); bacitracin (Serva: 1450 D); polymicin (Sigma: 1225 D). The SDS gels were either stained using the silver staining according to Morrissey (1981) or subdivided in 5 mm slices, which were eluted as described above for the PAGE gels.

Furthermore, the immunoreactive fraction of the sinus gland separated by PAGE was further analysed on HPLC, using the described reversed phase system.

Immunochemical detection

Eluted and lyophilized samples obtained after PAGE, HPLC and SDS electrophoresis were immunochemically tested applying the dotting immunobinding assay (DIA) according to Hawkes et al. (1982), in combination with the anti-*Astacus*-CHH serum. Nitrocellusose paper (Schleicher and Schüll; BA 85; 0.45 µm) was used as a carrier for the samples. The lyophilized samples were dissolved in distilled water and "dotted" on the paper (1 µl/"dot") using a calibrated 5 µl micropipet. After drying, the paper was placed in an appropriate vessel and the non-specific antibody binding sites were blocked by adding blocking solution (BS) for 15 min at room temperature (BS: 3% w/v bovine serum albumine or ovalbumine, 1% normal goat serum in Tris buffered saline (TBS): 50 mM Tris-HCl, 200 mM NaCl; pH 7.4). Further incubations were as follows: incubation with anti-*Astacus*-CHH serum in BS (dilution 1/400 4°C; 18 h), then BS (15 min, at room temperature) and finally goat-anti-rabbit peroxidase (GAR-PO: dilution 1/200 in BS; 2 h at room temperature). Between each incubation the paper was washed several times in TBS and all incubations took place under constant shaking. Visualization of the immunoreactive dots took place by incubation in a 3% solution of 4-Cl-1-Naphtol and 0.01% peroxide during 2-30 min. The density of the immunoreactive dots was quantified using a microspectrofotometer (Leitz Orthoplan). The specificity of the immunochemical reaction was tested by substituting normal rabbit serum for the primary antiserum. Preliminary experiments for the sensitivity in the

described DIA procedure yielded the following data: the CHH material present in 1/250th of a cell group, 1/750th of an axonal tract and 1/10000th of a sinus gland can be detected.

Determination of the biological activity

The obtained fractions after PAGE, HPLC and SDS electrophoresis were tested on hyperglycemic activity by injecting 100 µl samples of these fractions (dissolved in modified Van Harreveld's saline solution: see Chapter VI) in the crayfish hemolymph. The injected dose of perikarya group equivalents (PE), tract equivalents (TE) and sinus gland equivalents (SE) was determined according to the approach by Leuven et al. (1982) as described in Chapter VI. Blood was collected as described in Chapter IV. The hemolymph glucose content was determined using the GOD-Perid Methode (Boehringer Mannheim GmbH).
Both *Astacus leptodactylus* and *Orconectes limosus* were used for bioassays.

A flow chart of the succeeding steps in the analyses of the tissue samples is presented in Fig 1.

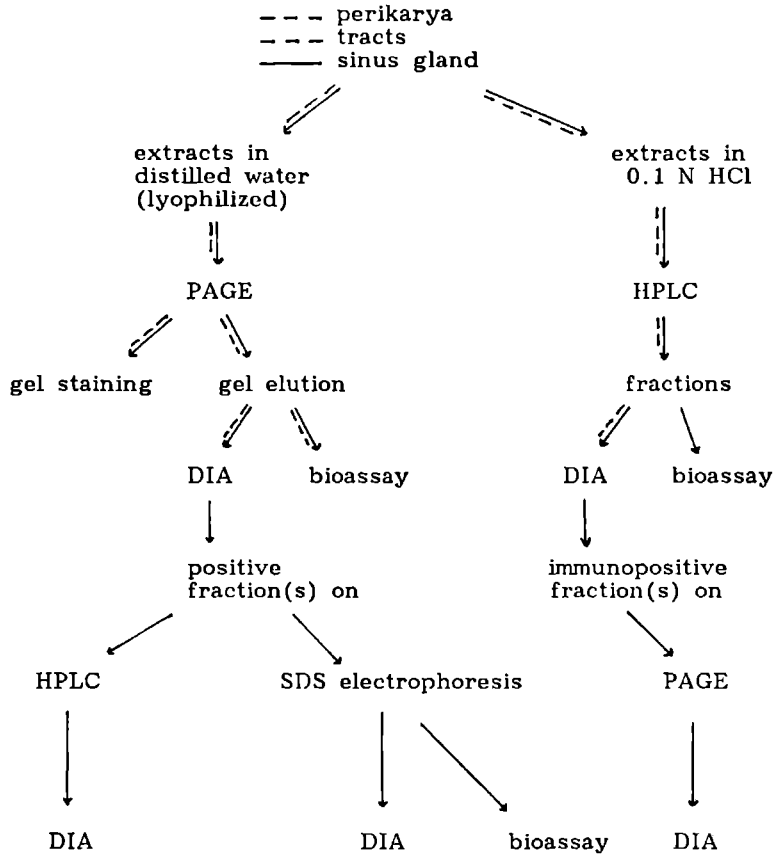

Figure 1. Diagram illustrating the successive steps in the analysis of crude extracts of CHH-producing perikarya, tracts and sinus glands.

Analyses of sinus gland extract

PAGE

The results obtained after PAGE of crude sinus gland extract, combined with the determinations on immunological and biological activity, are presented in Fig 2A-D. The analyses reveal hyperglycemic as well as anti-CHH immunological activity for the 5 mm gel slice which contains products with an Rm between 0.30-0.37 (Fig 2C,D). This fraction is associated with a rather broad and dense band with Rm 0.34 after gel staining with CBB (Fig 2A,B). This band could not be visualized by the silver staining.

The above determined hyperglycemic as well as immunopositive fraction was further analysed for molecular radius by use of SDS electrophoresis. After silver staining it was found that this zone is well separated from other proteins and that it consists of only one substance with Rm 0.6 (Fig 3). We calculate this product to correspond with a molecular weight of aproximately 7000 D. After SDS electrophoresis this product still reveals biological and immunological activity, although to a smaller extent.

HPLC

After separation on PAGE, the fraction with Rm 0.34 was further characterized by means of HPLC. The immunochemical analyses of the obtained HPLC fractions reveal an immunopositive reaction for a single peak eluting between 52-54 min (Fig 4).

Furthermore, characterization of crude sinus gland extract was performed directly by means of HPLC analysis. Two distinct immunoreactive peaks were found by DIA, the first eluting between 28-29 min and the second predominantly immunoreactive peak eluting between 52-54 min. A third slightly immunopositive fraction elutes at 56 min (Fig 5).

In view of the difference in HPLC elution time between the Rm 0.34 sinus gland fraction and the crude extract, we made a comparison by electrophoresis between the immunoreactive peaks obtained after HPLC analyses. After PAGE the anti-CHH positive products were found in the gel fraction with Rm 0.34 for both distinct HPLC peaks with an elution time of 28-29 or 52-54 min, respectively. The third weakly immunoreactive HPLC peak (elution time 56 min) was not observed after PAGE.

The tests for biological activity of the above-mentioned predominant HPLC peaks are not conclusive; elevation of blood glucose content was not always detectable.

Analyses of axonal tract extract

PAGE

Fig 6A-D shows the results after PAGE of crude axonal tract extract combined with the determinations on immunological and biological activity. The analyses reveal biological as well as immunological activity for the Rm=0.34 fraction (Fig 6C,D). The amount of protein was insufficient to allow detection after CBB staining (Fig 6A,B).

HPLC

The results after HPLC of crude axonal tract extract combined with the immunochemical determinations are presented in Fig 7. The results show two distinct immunopositive peaks. The first predominant immunoreactive peak elutes between 28-29 min. The second less immunopositive peak elutes after 52-54 min.

Analyses of perikarya extract

PAGE

The correlations between the immunological and biological activity after PAGE of crude perikarya extract are shown in Fig 8A-D. Also in this case, the analyses reveal biological as well as immunological activity for the fraction with Rm=0.34 (Fig 8C,D). Furthermore, a second bioactive product was detected in the 5 mm gel slice which contains products with an Rm between 0.01-0.07 (Fig 8C). However, this fraction did not show immunopositive reaction with the primary antiserum. The amount of protein was too small to yield a band after staining with CBB in the region with Rm 0.30-0.37, however we detected a faint band with Rm 0.06 (Fig 8A,B). Silver staining did not reveal any bands in both regions.

HPLC

The results of HPLC analyses and immunochemical determinations of perikarya extracts are presented in Fig 9. After DIA, three rather faint immunopositive regions were observed to elute at 28-29 min and 56 min.

After PAGE of crude sinus gland extract a single fraction is obtained with an electrophoretic mobility of Rm 0 34 and with both biological and immunological activity SDS- PAGE electrophoresis of this fraction reveals a single band with an apparent molecular weight of around 7000 D that is still biologically active and immunologically reactive to anti-CHH These data on the hyperglycemic material in the sinus gland of ***Astacus leptodactylus*** are in line with the results of Keller (1977) who found that the hyperglycemic material in Astacidea had an Rm value of 0 35 The apparent molecular weight of approximately 7000 D reported for the CHH of ***Astacus leptodactylus*** compares well with the values found for hyperglycemic material in the prawn ***Pandalus jordani***, (6300 D Kleinholz and Keller 1973), the shrimp ***Crangon crangon***, (7300 D Skorkowski et al 1977), the crab ***Carcinus maenas***, (6726 D Keller and Wunderer 1978), the crayfish ***Orconectes limosus***, (6812 D Keller 1981) and the land crab ***Cardisoma carnifex***, (6000 D Stuenkel 1983)

HPLC analyses of crude sinus gland extract yielded two predominant immunologically positive fractions and a third less distinct fraction The two predominant HPLC peaks demonstrate the same electrophoretic mobility after PAGE, which indicates a close relationship between these two peptides On the other hand, when the Rm 0 34 material after electrophoresis was further analysed by HPLC, we could only detect the peak with an elution time of 52-54 minutes This implies that the gel fraction is homogeneous and remains stable during HPLC, which is also observed after SDS electrophoresis These findings indicate the existence of two hyperglycemic factors in the sinus gland, detectable after HPLC but not after the applied electrophoretic analyses, that may be related via a prohormone or a precursor relationship Furthermore, electrophoresis may transform the different factors to one stable peptide However, we do not exclude the possibility that the presence of two factors is an artifact resulting from electrophoretic or chromatographic treatments In this respect, Stuenkel (1983) reported similar observations He found two predominant peaks with hyperglycemic activity after RPLC of sinus gland extract of ***Cardisoma carnifex***. The amino-acid compositions of these peaks, determined by Newcomb (1983), proved to be very similar The author proposed therefore that both had a related biosynthetic origin and that a charge difference or a difference in nonamino-acid components between the two peptides was the cause of their separation on RPLC After SDS electrophoresis Stuenkel (1983) described hyperglycemic activity for a predominant factor with a molecular weight of 6000 D and for a second factor of 11000 D which was not always observed However, he did not make further analyses by means of a combination between electrophoresis and RPLC

Our tests of the biological activity of the immunoreactive HPLC peaks did not give conclusive evidence. This may be caused by the conditions to which the hyperglycemic material is exposed during chromatography

PAGE of crude tract and CHH perikarya extracts revealed the same biologically and immunologically active gel fraction with Rm 0 34, as found in sinus gland extract Moreover, in perikarya extract a second factor with low electrophoretic mobility is found This factor, although not immunochemically detectable, may represent the prohormone or precursor of CHH Previous immunocytochemical investigations on the electron microscopic level revealed an immunopositive reaction in the perikarya for the primary neurosecretory CHH granules and the content of the Golgi-sacculi (Chapter I) This observation may indicate that the biologically active gel fraction with low electrophoretic mobility represents material that is not yet packed in granules, e g present in the cisternae of the rough endoplasmic reticulum However, it cannot be excluded that the fixation procedure for electron microscopic observations may be responsible for structural changes in the CHH molecule which result in

immunochemical detection of the hyperglycemic factor present in the primary granules. This biologically active gel fraction found in the cell bodies was not detected in the tract extract, which indicates that maturation of the granule content occurs in the perikaryon before the granules are transferred into the axons.

After analysis of tract and perikarya extracts on HPLC, the same immunopositive peaks are detected as described for the sinus gland extract. The shift of predominant immunological peaks in the axonal tract and the sinus gland may indicate further biochemical changes of the hyperglycemic material during transport. The low immunoreactivity of the perikarya extract suggests that the synthesized material is quickly transported or that the non-immunochemically detectable hyperglycemic factor still predominates in the cell body.

Molecular heterogeneity of the hyperglycemic hormone and the presence of a precursor or prohormone of the CHH have been reported by several authors for different species of decapod crustaceans applying variable detection methods (Andrew and Saleuddin 1979; Kleinholz and Keller 1973; Keller 1977; Skorkowski et al. 1977; Newcomb 1983; Stuenkel 1983; Van Wormhoudt et al. 1984a,b). The present results are in line with the above mentioned reports. However, many questions are left for further studies to illucidate the nature of these multiple forms, which may be the result of functional molecular variants, although artifacts due to the applied separation procedures cannot be excluded.

Fig. 1: Diagram illustrating the succeeding steps in analysing crude extracts of CHH-producing perikarya, tracts and sinus glands.

Fig. 2A-D: Results obtained after PAGE of sinus gland extracts.
A: gel after electrophoresis of 10 SE, CBB staining;
B: drawn electropherogram of the gel in A;
C: hyperglycemic respons after injection of 1 SE/animal, means ±S.E.M., n=5.
D: immunological reaction quantified by microspectrofotometry for 0.08 SE/dot.

Fig. 3: SDS gel after electrophoresis of the Rm 0.34 gel fraction (after PAGE), 6 SE, silverstaining.

Fig. 4: Results of the DIA for the HPLC fractions of the Rm 0.34 gel fraction (after PAGE), 0.1 SE/dot.

Fig. 5: Results of the DIA for the HPLC fractions of sinus gland extract, 0.1 SE/dot.

Fig. 6A-D: Results obtained after PAGE of tract extract.
A: gel after electrophoresis of 30 TE, CBB staining;
B: drawn electropherogram of the gel in A;
C: hyperglycemic respons after injection of 300 TE/animal; means ±S.E.M., n=5.
D: immunological reaction quantified by microspectrofotometry for 3 TE/dot.

Fig. 7: Results of the DIA for the HPLC fractions of tract extract, 5 TE/dot.

Fig. 8A-D: Results obtained after PAGE of sinus gland extract.
A: gel after electrophoresis of 30 PE, CBB staining;
B: drawn electropherogram of the gel in A;
C: hyperglycemic response after injection of 150 PE/animal; means ±S.E.M., n=5.
D: immunological reaction quantified by microspectrofotometry for 2 PE/dot.

Fig. 9: Results of the DIA for the HPLC fractions of perikarya extract, 4 PE/dot.

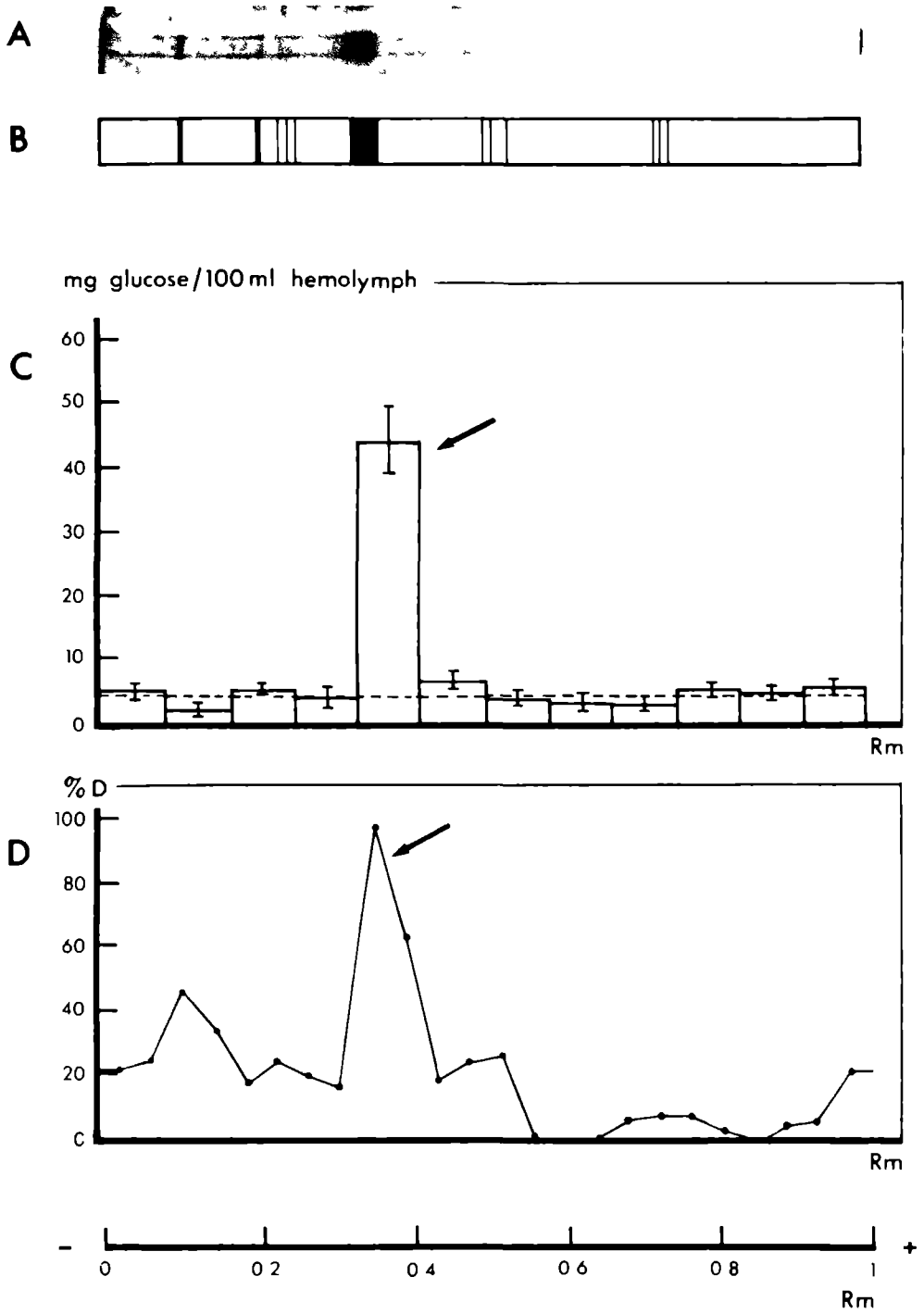
A
B
C
mg glucose/100 ml hemolymph
60
50
40
30
20
10
0
Rm
D
% D
100
80
60
40
20
0
Rm
-
+
0
0 2
0 4
0 6
0 8
1
Rm

fig. 2 A-D

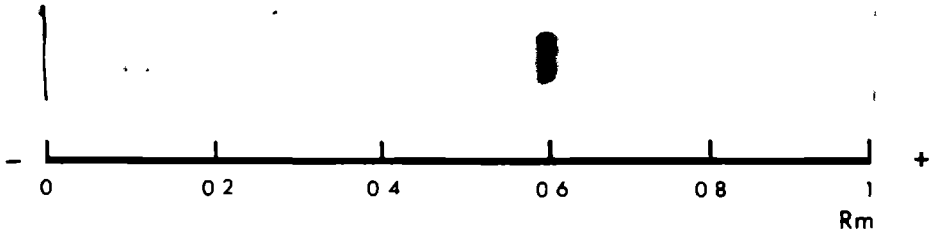

fig. 3

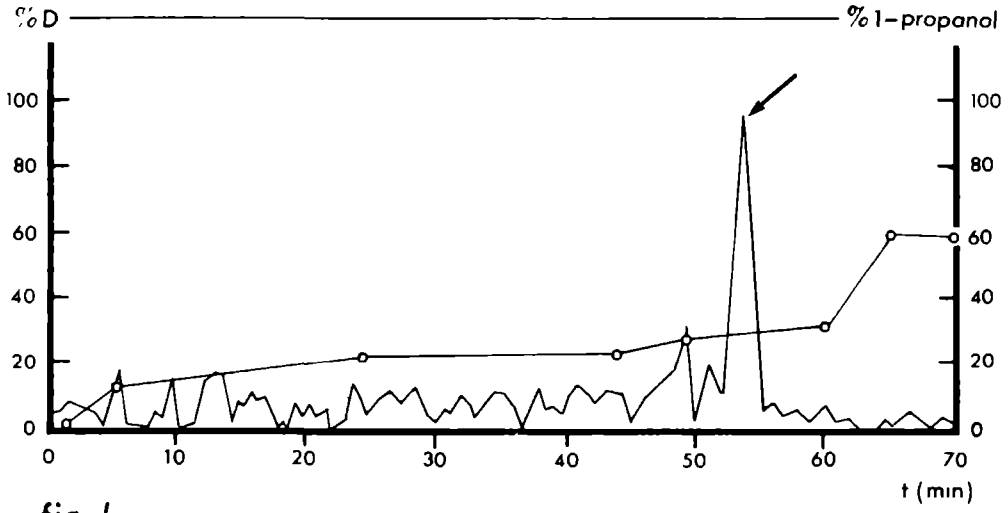

fig. 4

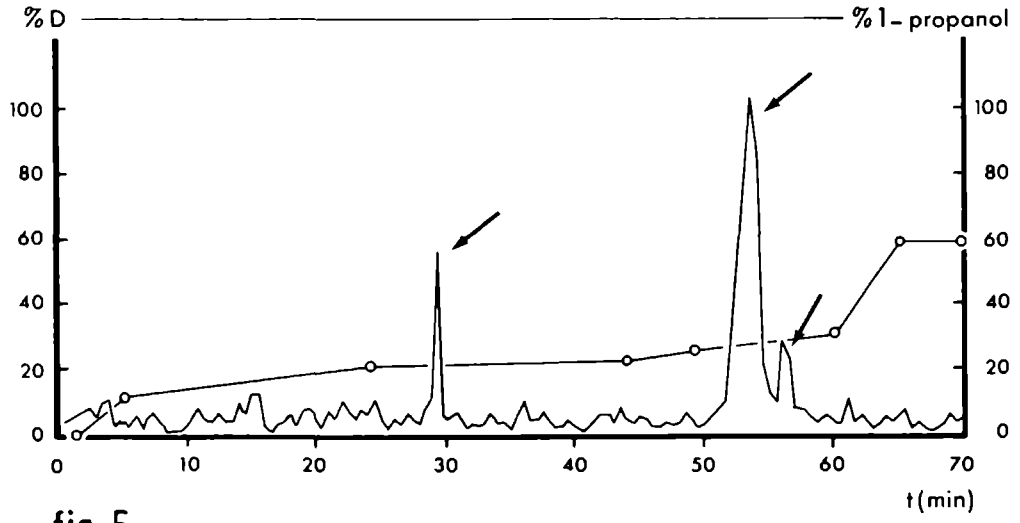

fig. 5

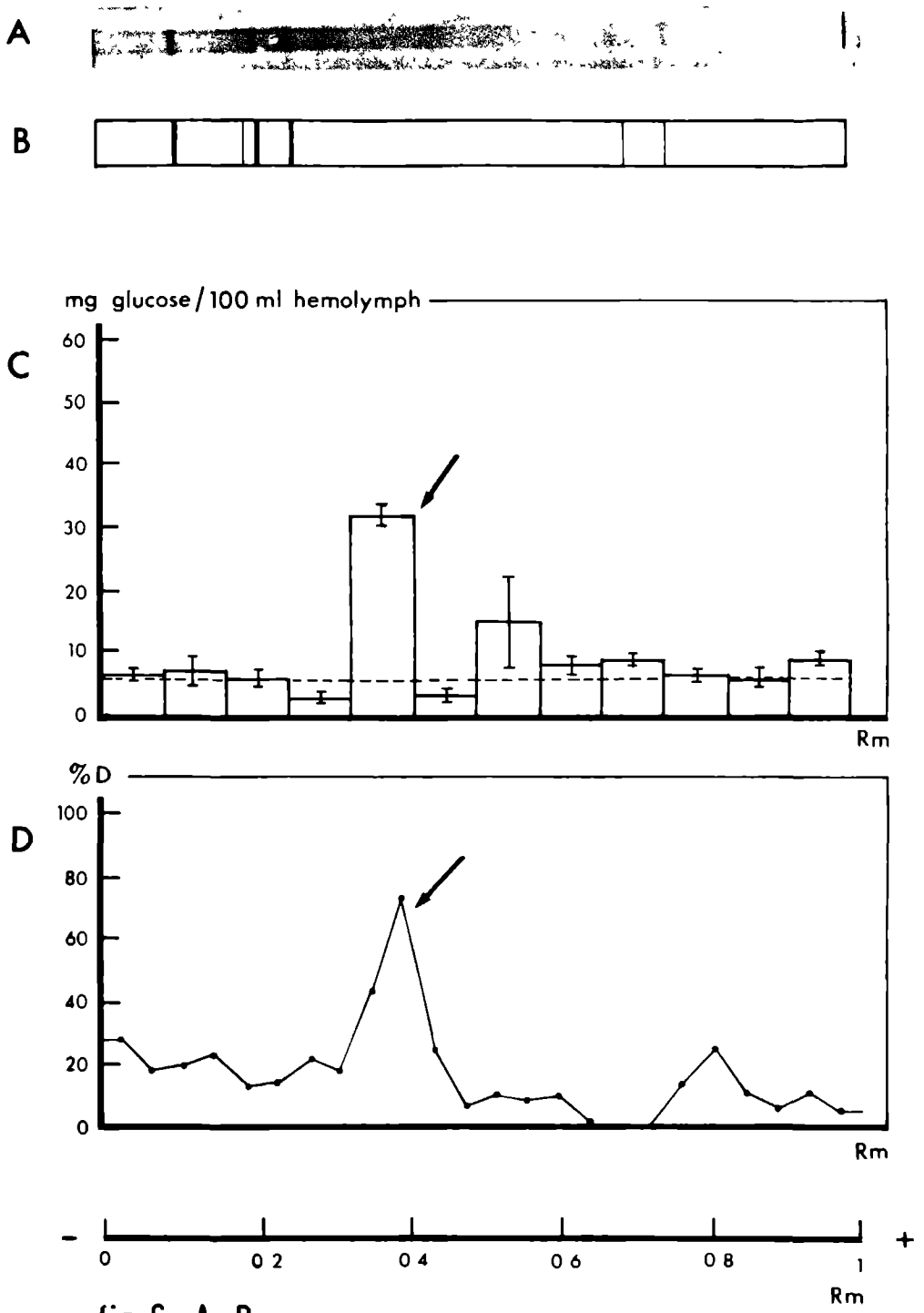
A
B
C
mg glucose / 100 ml hemolymph
60
50
40
30
20
10
0
Rm
D
%D
100
80
60
40
20
0
Rm
-
+
0
0 2
0 4
0 6
0 8
1
Rm

fig. 6 A-D

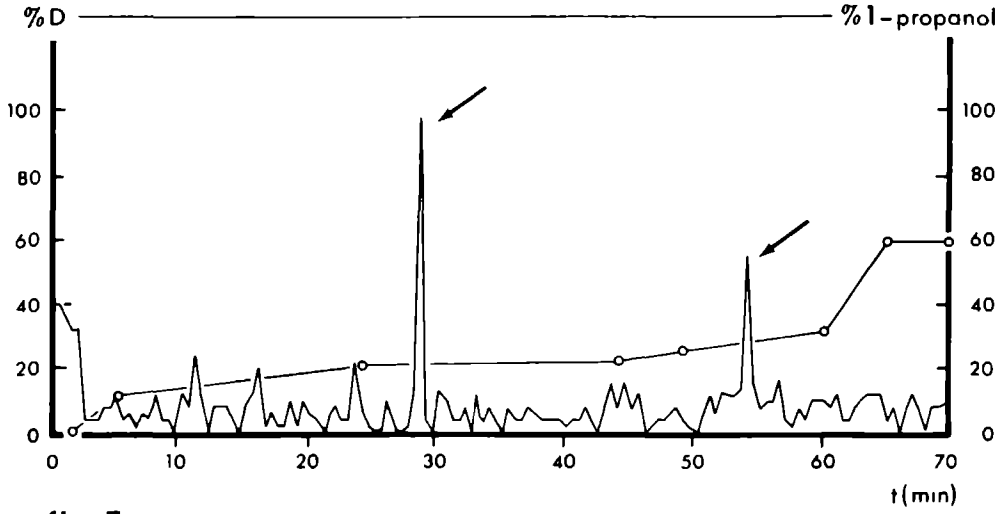
%D
%1-propanol
100
80
60
40
20
0
0
10
20
30
40
50
60
70
t(min)

fig. 7

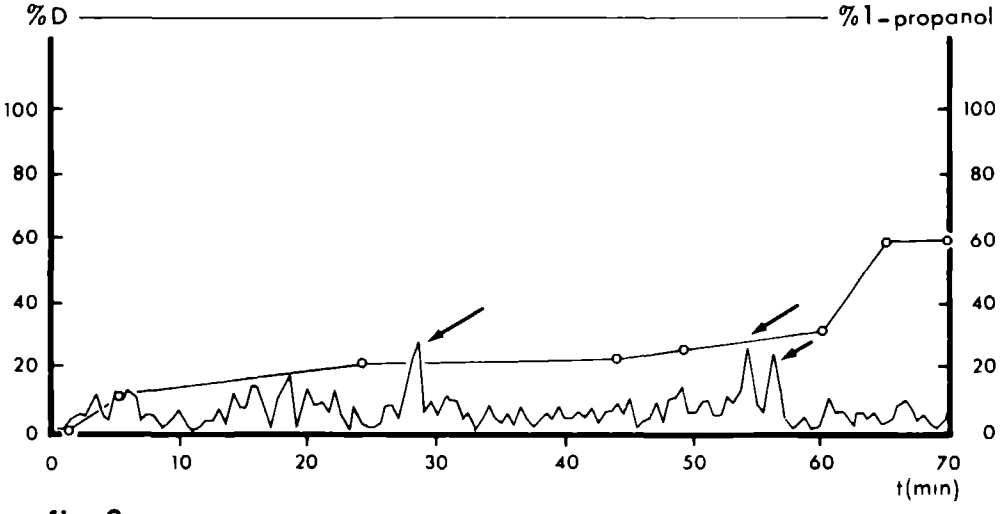
%D
%1-propanol
100
80
60
40
20
0
0
10
20
30
40
50
60
70
t(min)

fig. 9

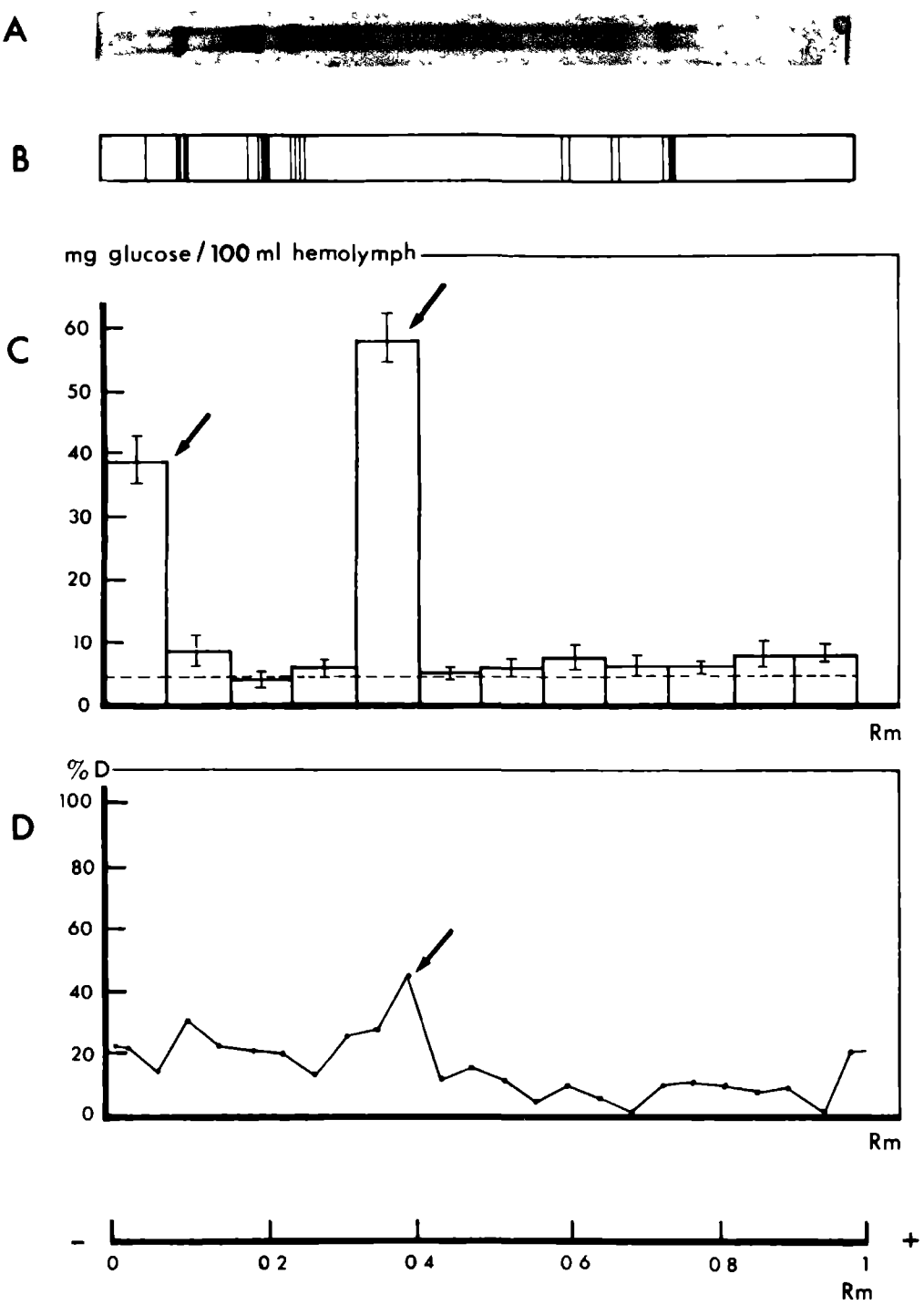
A
B
C
mg glucose / 100 ml hemolymph
60
50
40
30
20
10
0
Rm
D
% D
100
80
60
40
20
0
Rm
-
+
0
0 2
0 4
0 6
0 8
1
Rm

fig. 8 A-D

Most of the results presented in this thesis are based on the immunocytochemical detection (PAP method) of the crustacean hyperglycemic hormone (CHH)-producing neurosecretory system in the eyestalk of the crayfish ***Astacus leptodactylus*** (Nordmann 1842), using an anti-***Astacus***-CHH serum

The CHH-producing cells form part of the medulla terminalis X-organ (MTGX), where they are found as a distinct group, consisting of about 35 cells, situated at the rostro-distal part, latero-ventrally on the MTGX Their axons form a tract which traverses the medulla terminalis neuropil and runs to the sinus gland Here the axons split up into numerous axon terminals By light and fluorescence microscopy the CHH is visible as granular material randomly distributed throughout the cytoplasm Electron microscopy reveals that the immunopositive reaction occurs in Golgi sacculi and in the CHH-containing granules present in the cell body, the axon and the axon terminals (Chapter I)

In Chapter II a comparative study is presented on the location of the CHH system in the eyestalk of various decapod species The CHH cells of all species investigated are found in a similar region as described for ***Astacus***. These results indicate that the sites of production, storage and release of CHH are comparable for all infraorders studied, the Astacidea, Brachyura, Caridea and Palinura

After immunocytochemical staining on the light microscopic level, the CHH cells demonstrate striking individual differences in intensity of immunostaining In this respect we made an arbitrary subdivision of the cells in +, ++ and +++, indicating increasing immunoreactivity, respectively Morphometric analyses at the light and electron microscopic level indicate that these differences in immunostaining are correlated with differences in numerical density of the CHH granules in the cytoplasm a weakly immunopositive cell (+) contains less granules than a strongly immunopositive cell (+++) Furthermore, between these three cell stages considerable difference in cellular and nuclear volumes and in the number and volumes of cell organelles are observed Based on these data we present a description of the synthetic activity levels of the CHH cells Furthermore, we observed changes in electron density and diameter of the CHH granules older stored granules are less electron dense and increased in diameter compared to newly synthesized primary granules This points to a maturation process of the CCH granules (Chapter III)

In the above study immunostaining is introduced as a method to obtain information on the secretory activity of individual CHH cells This method was further used to study the secretory dynamics of the CHH cell group during a 24 h cycle (constant 12 h light/12 h dark) The changes of the cytoplasmic and nuclear volumes of the CHH cells are correlated with the exocytotic activity of the sinus gland and the nocturnal rise in hemolymph glucose concentration The obtained data lead to the hypothesis that CHH production is stimulated two hours before the peak of CHH release in the sinus gland Four hours later this peak is followed by hyperglycemia (Chapter IV)

The same immunostaining method is applied to obtain information on the secretory dynamics of the CHH system during the moulting period The results show a firmly reduced secretory activity of the CHH cells for all moulting stages In addition, examinations on the 24 h blood glucose rhythmicity reveals the complete absence of the nocturnal peak Furthermore, we observed that ecdysteroids can affect the daily blood glucose rhythm and may function as a pheromone Supply of β-ecdyson to the water results in the disappearance of the nocturnal blood glucose peak In addition, intermoult animals brought in contact with animals going through moult, do not demonstrate nocturnal hyperglycemia (Chapter V)

Examination of the larval and early postlarval stages reveal immunopositive cells already in the larval stage immediately after hatching. During postlarval development the number of immunopositive cells increases. Ultrastructural investigation of the sinus gland reveals axon terminals filled with immunoreactive CHH granules. During postlarval development the mean diameter of these granules increases. Injection of larval eyestalk extract in adult crayfish causes a rise in blood glucose level (Chapter VI).

The location of the CHH cells, distally in the MTGX, enables us to inject single cells with a microelectrode. In this way individual CHH cells are traced iontophoretically with the fluorescent dye Lucifer Yellow. This results in the visualization of the total course of the axon, which reveals many fine processes originating in the proximal part of the axon. The arborizations protrude into the medulla terminalis neuropil. Ultrastructural observation shows that these CHH processes are densily packed with neurosecretory granules. On the axon ramifications synaptic input is observed from neuronal processes containing both haloed dense-cored granules and clear vesicles (Chapter VII).

In chapter VIII a pilot study is presented on the molecular nature of the CHH as it occurs in the perikarya, the tract and the sinus gland, respectively. The hyperglycemic material is analysed by polyacrylamide gel electrophoresis (PAGE), SDS-PAGE and high pressure liquid chromatography (HPLC), in combination with immunochemical and biological detection. By means of PAGE and SDS-PAGE a predominant immunoreactive and biologically active hyperglycemic factor is found in the cell body, the tract and the sinus gland, with an average molecular weight of around 7000 D. In extracts of the perikarya, a second biologically active fraction is found with low electrophoretic mobility. The latter may represent the prohormone or precursor of CHH. By means of HPLC two predominant peaks are determined corresponding with an elution time of 28-29 min and 52-54 min, respectively.

HET HYPERGLYCEMISCH HORMOON PRODUCEREND SYSTEEM IN DE OOGSTEEL VAN DE KREEFT ASTACUS LEPTODACTYLUS

Het crustaceeen hyperglycemisch hormoon (CHH) wordt geproduceerd in een aantal neurosecretorische cellen gelegen in de oogsteel van decapode crustaceeen Met behulp van een anti-*Astacus* CHH serum en een immunocytochemische kleuring is het CHH gelocaliseerd in het X-orgaan sinusklier complex van de moeraskreeft *Astacus leptodactylus* (Nordmann 1842) Het CHH systeem bestaat uit een groep van ongeveer 35 cellen die deel uit maken van het medulla terminalis ganglionair X-orgaan (MTGX), gelegen rostro-distaal aan de latero-ventrale zijde van de medulla terminalis De axonen van deze cellen bundelen zich tot een tractus die door de medulla terminalis heen loopt naar het neurohemaal gebied, de sinusklier, waar de axonen zich vertakken in een groot aantal axoneindigingen Na de immunocytochemische kleuring is het CHH, met behulp van het licht- en fluorescentiemicroscoop, zichtbaar in de vorm van klompjes immunopositief materiaal willekeurig verspreid over het cytoplasma Bekijken we de ultrastructuur van de immunoreactieve CHH cellen, dan zien we dat de immuunreactie optreedt in de sacculi van de Golgi complexen en in de neurosecretorische granula gelegen in het perikaryon, het axon en de axoneindigingen (Hoofdstuk I)

Met behulp van het anti-*Astacus*-CHH serum is een vergelijkend onderzoek verricht naar de ligging van de CHH cellen in de oogsteel van een aantal vertegenwoordigers van verschillende infraorden Astacidea, Brachyura, Caridea en Palinura De resultaten tonen een overeenkomstige ligging bij alle onderzochte soorten zodat algemene conclusies getrokken kunnen worden ten aanzien van de localisatie van het CHH-producerend systeem bij decapode crustaceeen (Hoofdstuk II)

Na immunocytochemische kleuring vertonen de CHH cellen op lichtmicroscopisch niveau grote onderlinge verschillen ten aanzien van de intensiteit van de immuunreactie Dit heeft geleid tot het maken van een semi-kwantitatieve indeling op grond van oplopende kleurintensiteit in +, ++ en +++ cellen Het bestuderen van de ultrastructuur van deze cellen leidde tot de conclusie dat de verschillen in kleurintensiteit gecorreleerd zijn aan verschillen in dichtheid van de CHH granula in het cytoplasma een zwak immunopositieve cel (+) bevat veel minder granula dan een sterk immunopositieve cel (+++) Daarnaast zijn er aanzienlijke verschillen aangetoond in het cel- en kernvolume en in het aantal en het volume van celorganellen Bovendien zijn er aanwijzigingen gevonden voor het optreden van een rijpingsproces van het hyperglycemisch materiaal binnen de granula Naar mate de weg die de granula moeten afleggen vanaf de afsnoering van het Golgi complex tot de axoneindiging in de sinusklier vordert, neemt hun electronendichtheid af en hun diameter toe Op grond van deze gegevens is een beschrijving gemaakt van het verloop van de synthesecyclus van de CHH cellen (Hoofdstuk III)

In hoofdstuk III is aangetoond dat de intensiteit van de immuunreactie een maat is voor de synthesefase waarin de CHH cel zich bevindt Deze benadering is dan ook gekozen voor het verkrijgen van meer inzicht in het verloop van de synthese-activiteit van de CHH cellen tijdens een dag/nacht cyclus De proefdieren waren aangepast aan een constante 12 uur licht en 12

uur donker cyclus. Het verloop van het glucose gehalte in de hemolymfe vertoont onder deze omstandigheden een dag/nacht ritmiek waarbij er vier uur na het begin van de donkerperiode een piek in het glucosegehalte optreedt. De morfometrische gegevens hebben geleid tot de veronderstelling dat de productie van CHH en de afgifte ervan uit het perikaryon naar de axonen zes uur voordat het glucose gehalte in de hemolymfe maximaal is, extra gestimuleerd wordt (Hoofdstuk IV).

Op dezelfde wijze is informatie verkregen omtrent het verloop van de synthese-activiteit van de CHH cellen tijdens de vervellingscyclus. Bepalingen ten aanzien van de dag/nacht ritmiek van het glucose gehalte in de hemolymfe van kreeften in verschillende fasen van het vervellingsproces, laten zien dat de glucosepiek tijdens de donkerperiode volledig verdwijnt. Op grond van morfometrische resultaten kan geconcludeerd worden dat de synthese-activiteit van de CHH cellen sterk daalt tijdens het verloop van de vervellingsperiode. Daarnaast zijn aanwijzingen verkregen dat β-ecdyson een modulerende invloed heeft op de dag/nacht ritmiek van de bloed suiker spiegel en dat deze stof en hieraan verwante ecdysteroiden als feromoon kunnen functioneren. Toevoegen van β-ecdyson aan het milieu resulteert in het verdwijnen van de nachtelijke glucose piek. Deze piek verdwijnt eveneens bij kreeften verkerend in het tussenvervellingsstadium die in contact gebracht worden met dieren die een vervelling doormaken (Hoofdstuk V).

In hoofdstuk VI worden de resultaten weergegeven van onderzoek naar het voorkomen van het CHH-producerend systeem tijdens de larvale en postlarvale ontwikkeling. Vanaf het larvale stadium, onmiddelijk na het uitkomen van de eieren, zijn immunopositieve cellen aanwezig en worden immunopositieve granula in de sinusklier aangetroffen. Tijdens het verloop van de postlarvale ontwikkeling neemt het aantal immunoreactieve cellen en de diameter van de immunopositieve granula toe.

Vanwege hun grootte en hun distale ligging in het MTGX, kunnen individuele CHH cellen met behulp van een micro-electrode worden aangeprikt. Op deze manier zijn CHH cellen electroforetisch gevuld met de fluorescerende stof Lucifer Yellow, waarbij het verloop van een axon van een cel tot in de sinusklier zichtbaar gemaakt wordt. Deze methode heeft aan het licht gebracht dat het proximale deel van het axon zich in het neuropileum van de medulla terminalis vertakt. Electronenmicroscopisch onderzoek laat zien dat op deze vertakkingen informatie-overdracht plaats vindt in de vorm van synapsen (Hoofdstuk VII).

In hoofdstuk VIII wordt verslag gedaan van een oriënterende studie over de moleculaire structuur van het CHH zoals het voorkomt in de perikarya, in de axonen en in de sinusklier. Het hyperglycemisch materiaal is geanalyseerd met behulp van polyacrylamide gel electroforese (PAGE en SDS-PAGE) en hoge druk vloeistof chromatografie (HPLC), gecombineerd met een immunochemische en een biologische toets. Met behulp van PAGE en SDS-PAGE is een immunopositieve en hyperglycemisch actieve factor aangetoond in zowel perikaryon, tractus en sinusklier-extract, met een molecuulgewicht van ongeveer 7000 D. In het perikaryon-extract is bovendien een tweede biologisch actieve factor aangetoond met een lagere electroforetische mobiliteit; dit wijst op een stof met een groter molecuulgewicht. Wellicht hebben we hier te maken met een prohormoon of precursor van het CHH. Met behulp van HPLC zijn twee immunopositieve pieken gevonden met een elutietijd van respectievelijk 28-29 min en 52-54 min.

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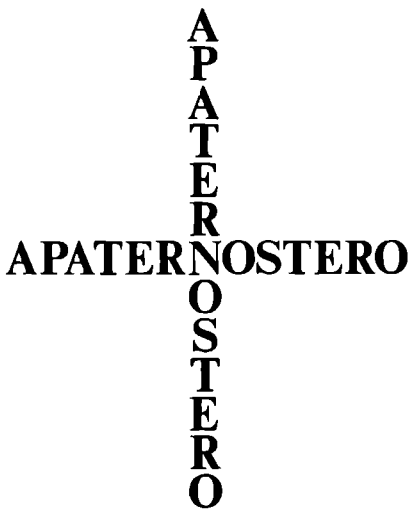
Janine Louise Kallen (e.v. Gorgels) werd op 6 september 1955 geboren te Maastricht. Aldaar doorliep zij de lagere school (St. Alfonsusschool, Bosserveld). In 1970 werd het MULO diploma behaald (St. Carolus MULO, thans MAVO Biesland) en in 1972 het h.b.s.-A diploma (Jeanne d'Arc lyceum, thans Scholengemeenschap Jeanne d'Arc). Van 1972 tot 1973 was zij ingeschreven als toehoorder aan de Rijksuniversiteit Utrecht waar zij een avondopleiding volgde ten behoeve van de bijscholing in de B-vakken (Voorbereidende Cursus, Dr. Blaauw). In september 1973 werd begonnen met de studie biologie aan de Rijksuniversiteit Utrecht. In juni 1979 werd het MO-examen (met onderwijsbevoegdheid), het kandidaatsexamen (B1) en het doctoraalexamen afgelegd. Het doctoraalexamen omvatte het hoofdvak experimentele embryologie (Dr. J.A.M. van den Biggelaar), het bijvak electronenmicroscopische structuuranalyse (Dr. P.H.J.Th. Ververgaert) en de nevenrichtingen algemene plantenfysiologie (Dr. H.W. Groeneveld) en didactiek van de biologie (C. Koetsier).
Van 1 september 1979 tot 1 augustus 1984 was zij aangesteld als wetenschappelijk medewerkster aan het Zoölogisch Laboratorium van de Katholieke Universiteit te Nijmegen. Tijdens deze periode is een bijdrage geleverd aan het prae- en postkandidaatsonderwijs biologie en scheikunde (anatomie van vertebraten aan eerstejaars studenten, zoölogie aan derdejaars studenten, vergelijkende endocrinologie van evertebraten aan derdejaars studenten, immuunbiologie aan derdejaars studenten, ontwikkelingsbiologie aan derdejaars studenten, stagebegeleiding van doctoraalstudenten).
Momenteel is zij als wetenschappelijk medewerkster verbonden aan het Zoölogisch Laboratorium van de Katholieke Universiteit te Nijmegen.

Bij de omslag:

Een ***kreeftdicht*** of ***retrograde*** is een gedicht samengesteld uit versregels waarvan de woorden evengoed grammaticaal correcte zinnen vormen wanneer ze van achteren naar voren worden gelezen. Deze zeer gekunstelde en onnatuurlijke dichtvorm was met name in zwang onder de middeleeuwse rederijkers. Een ***palindroom*** is een woord, zin of vers waarvan de letters van achteren naar voren gelezen kunnen worden, waarbij de betekenis al dan niet verandert. Het palindroom wordt bij voorkeur gebruikt in kreeftdichten. In vroeger eeuwen werd het procédé vaak aangewend als tover- of bezweringsformule, zoals het magisch vierkant
"sator-arepo-tenet-opera-rotas".
Dit kreeftdicht heeft een zeer wijde verspreiding, in plaats, tijd en toepassing. De oudste inscriptie is gevonden in Pompeï, dus voor 79 na Christus geschreven. Het duikt onder andere op rond 1600 in IJsland en in 1742 in Sachsen-Weimar.
Over de betekenis van de spreuk lopen de meningen uiteen: letterlijk vertaald staat er "de zaaier Arepo houdt met kracht de wielen vast". Een christelijke interpretatie is mogelijk: in het palindroom vormt het woord ***tenet*** een kruis, voorts is het mogelijk anagrammatisch van de spreuk een kruis te vormen waarvan de balken bestaan uit de letters alfa en omega en de eerste twee woorden van het Onze Vader:



Men vermoedt dan ook dat de spreuk gebruikt is door de eerste christenen om zich als zodanig aan medechristenen kenbaar te maken, terwijl de betekenis voor derden verborgen bleef.

Daarnaast zijn interpretaties mogelijk die meer naar magie en zwarte kunst neigen. Zo kan ook gelezen worden: "satan operor te, operor te satan" (o satan, satan, aan uw werk neem ik deel), of: "satan, oro te, pro arte a te spero" (satan, tot u richt ik mijn smeekbeden, op uw toverkunst reken ik), of: "satan ter oro te, opera praesto" (satan, ik bid u driewerf en verricht uw werken). In deze trant zijn meer (vergezochte?) anagrammen gemaakt.

De toepassingen zijn al even uiteenlopend. In een christelijke context wordt de spreuk gebruikt in een dertiende eeuws handschrift over geneeskrachtige middelen en spreuken, waarin aangeraden wordt: "...pour une femme, qui est en mal d'enfant liez cet écrit sur le ventre: Maria peperit Christum, Anna Mariam,..... sator arepo tenet opera rotas."
Bij het bezweren van de duivel werd hij op een bijzondere manier gebruikt: mensen die in verband gebracht werden met tovenarij, kregen gedurende negen dagen een dieet van duivelsdrek en brood, uitgesneden in de 25 letters van het palindroom.
De sator-arepo spreuk werkte ook preventief: op een briefje geschreven en aangebracht op luiken en deuren zou hij diefstal voorkomen.
Van de preventieve werking werd ook middels toverspreuken gebruik gemaakt:
-om niet over een steen te vallen:
lees de sator-arepo spreuk gevolgd door:
"qui timentis te, videbunt me,
qui in verbo tuo supersperavi". (Ps. 119,74).
-om geen schade aan gewassen te krijgen:
lees de sator-arepo spreuk gevolgd door:
"ne me demergat tempestas aquae
neque absorbeat me profundum,
neque urgeat super me patens os suum". (Ps. 69,16).
-om kwaad door brand te voorkomen:
lees de sator-arepo spreuk, sla een kruisteken en zeg:
"fiat misericordia tua ut consolet
me secundum elogum tuum, servo tuo". (Ps. 119,76).
Deze spreuken zijn citaten uit de toverboeken van
Jón Gudmundsson (1574-1650).

Voor meer informatie over het gebruik van dit magisch vierkant zij verwezen naar het orgaan van het Nederlands Klassiek Verbond
o.a. Hermeneus 53, nr 1 (1981).

STELLINGEN

I

De toename in diameter gecorreleerd met de afname in electronendichtheid van de CHH granula van ***Astacus leptodactylus*** tijdens hun transport van de plaats van productie naar de plaats van afgifte, zijn aanwijzingen voor het optreden van veranderingen in de biochemische structuur van het hyperglycemisch materiaal.

Dit proefschrift.

II

De intensiteit van de immunocytochemische kleuring van individuele CHH cellen van ***Astacus leptodactylus*** wordt bepaald door de hoeveelheid CHH granula in het cytoplasma en geeft informatie omtrent de synthese fase van de CHH cel.

Dit proefschrift

III

Het verdient aanbeveling de rol te onderzoeken die de chemoreceptoren in de laterale antenne spelen bij de invloed van in het milieu aanwezige ecdysteroiden op het functioneren van het CHH systeem

Spencer M and Case JF (1984), J Exp Zool 229: 163-166.

Dit proefschrift

IV

Gezien de belangrijke functies van prolactine voor de hydromineraalhuishouding bij aquatische lagere vertebraten, is het raadzaam meer aandacht te schenken aan de rol van prolactine bij de osmoregulatie tijdens de foetale periode.

Golander A et al (1978), Science 202 311-313.
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Talamantes F et al. (1980), Federation Proc 39. 2582-2587.
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Markoff E et al. (1982), J Endocrinol 92. 103-110.

V

De resultaten uit het onderzoek van Scheller en collega's aangaande de gecoördineerde physiologische functies van verschillende peptiden afkomstig van een gemeenschappelijke precursor bij ***Aplysia***, stimuleren onderzoek naar de vraag of deze functionele coördinatie voor meerdere precursor-product relaties van toepassing is.

Scheller RH and Axel R (1984), Scientific Am 50: 44-52.

VI

In de bioassay die Quackenbush en Herrnkind gebruiken om simultaan het Gonaden Inhiberend Hormoon en het Vervellings Inhiberend Hormoon aan te tonen, wordt onvoldoende rekening gehouden met het antagonisme tussen voortplanting en somatische groei bij decapode crustaceeën.

Quackenbush LS and Herrnkind WF (1981), Comp Biochem Physiol 69A: 523-527.

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Webb HM (1977), Biol Bull 153: 630-642.

VII

In zijn beschrijving van de embryonale ontwikkeling van de jaarvis ***Nothobranchius guentheri*** geeft Peters een onjuiste interpretatie van zijn waarnemingen tijdens stadium Ib.

Peters N jr (1963), Int Rev Ges Hydrobiol 48: 257-313.

Wourms JP (1972); J Exp Zool 182: 143-168.

VIII

De aanwezigheid van hexagonaal gegroepeerde partikels en de afwezigheid van partikelrozetten op de PF-zijde van het plasmamembraan in de wortelhaarcellen van ***Lepidium*** zijn hoogstwaarschijnlijk het gevolg van de voorbehandeling met glycerol of sucrose.

Emons AC, Planta, in press.

Volkmann D, Planta, in press.

IX

De tegenstrijdigheden in de resultaten die Nishida en Satoh enerzijds en Meedel en Whittaker anderzijds hebben verkregen met betrekking tot de embryonale herkomst van de larvale spiercellen van ***Ciona intestinalis***, kunnen zeer wel het gevolg zijn van onvolkomenheden in de bruikbaarheid van horse radish peroxydase voor cell-lineage onderzoek

Nishida H and Satoh N (1983),
Developm Biol 99 382-394
Meedel TH and Whittaker JR (1984),
Developm Biol 105 479-487

X

Sedert men de "Mean Corpuscular Hemoglobin Concentration" (MCHC) berekent met behulp van een met optische en electrische detectie-systhemen gemeten "Mean Corpuscular Volume" (MCV), blijkt de MCHC een natuurconstante en ongeschikt als indicator van ijzergebrek bij de mens

Fischer SL and Fischer SP (1983),
Arch Intern Med 143 282-283
Personal communication, JPMC Gorgels

XI

Nu duidelijk wordt dat β-thalassemie in bepaalde gebieden van Nederland in betrekkelijk hoge frequentie voorkomt, mag men aannemen dat zuidelijke legers in de loop der eeuwen hun genetisch materiaal actief onder de nederlandse bevolking hebben verbreid

Van Waveren Hogervorst GD (1984),
Technicon Hematology Colloquium Zeist
Personal communication, JPMC Gorgels

XII

De ingrijpende renovatie van de benedenstad van Nijmegen is vergelijkbaar met de explosie van de bouw in baksteen in de 14e eeuw, enerzijds maakt zij deel uit van een historisch bouwproces, anderzijds vernietigt zij in grote mate het bodemarchief en daarmee alle oudere nederzettingssporen

Rijksdienst voor het Oudheidkundig Bodemonderzoek,
Jaarverslag 1979 58-62, 1980 44-47, 1981 46-48, 1982 49-51

XIII

Het veurkomme vaan herdisse in de Werreke hoof neet te veurkomme tot in de Werreke gewerrek weurt.
Personal communication, JFM Gorgels.

XIV

Het feit dat in weeroverzichten gesproken wordt van "geen regen van betekenis", doet vermoeden dat medewerkers van het KNMI zelden of nooit wasgoed buiten te drogen hangen.

J.L. Kallen, Nijmegen, 17 januari 1985.